Journal of Cellular Biochemistry 88:1129–1144 (2003)

Progression of Chondrogenesis in C3H10T1/2 Cells is Associated With Prolonged and Tight Regulation of ERK1/2

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Abstract Close contact of mesenchymal cells in vivo and also in super dense micromass cultures in vitro results in cellular condensation and alteration of existing cellular signaling required for initiation and progression of chondrogenesis. To investigate chondrogenesis related changes in the activity of ubiquitous cell signaling mediated by mitogen-activated protein kinases (MAP kinase), we have compared the effect of cell seeding of pluripotent C3H10T1/2 mesenchymal cells as monolayers (non-chondrogenic culture) or high density micromass cultures (chondrogenic) on the regulation and phosphorylation state of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and also on regulation of ERK1/2 nuclear targets, namely, activation protein-1 (AP-1) and serum response factor (SRF). Increasing cell density resulted in reduced DNA binding as well as activity of AP-1. SRF activity, on the other hand, was up-regulated in confluent monolayer cultures but like AP-1 was inhibited in micromass cultures. Low levels of PD 98059 (5 μM), a specific inhibitor of ERK1/2, resulted in delayed induction of AP-1 and SRF activity whereas higher concentrations of this inhibitor (10–50 μM) conferred an opposite effect. Increasing concentrations of the PD 98059 inhibitor in long term monolayer or micromass cultures (2.5 day) resulted in differential regulation of c-Fos and c-Jun protein levels as well as total expression and phosphorylation levels of ERK1/2. PD 98059 treatment of C3H10T1/2 micromass cultures also resulted in up-regulation of type IIB collagen and *Sox9* gene expression. While high expression of aggrecan and type IIB collagen genes were dependent on BMP-2 signaling, ERK inhibition of BMP-2 treated micromass cultures resulted in reduced activity of both genes. Our findings show that the activity of ERK1/2 in chondrogenic cultures of C3H10T1/2 cells is tightly controlled and can cross interact with other signaling activities mediated by BMP-2 to positively regulate chondrogensis. J. Cell. Biochem. 88: 1129–1144, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: AP-1; SRF; ERK1/2; PD 98059; BMP-2; C3H10T1/2 mesenchymal cells; chondrogenesis; micromass cultures

Cellular condensation is an early developmental stage in chondrogenesis of long bones which is initiated by aggregation or close juxtapositioning of pre-chondrogenic mesenchymal cells in vivo. This close cell contact is also required for chondrogenesis of primary cells in vitro and is generated by seeding cells at very high density as micromass cultures [Ahrens et al., 1977; Solursh, 1983, 1989; Maini and Solursh, 1991; Reddi, 1994]. In this study, our aim was to investigate the effect of close juxtapositioning of cells in micromass cultures on the ubiquitous signaling activities and their potential significance in initiation and/or maintenance of chondrogenesis. Due to its ease of maintenance in culture and capability to be genetically manipulated, we have selected the multipotential mouse embryonic C3H10T1/2 cell line [Taylor and Jones, 1979] as an in vitro model system for study of early cell signaling

Grant sponsor: NIH; Grant number: DE12864.

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Received 14 November 2002; Accepted 15 November 2002
DOI 10.1002/jcb.10458

changes leading to cellular condensation. Previously, we showed that like primary chick limb bud mesenchyme, C3H10T1/2 cells can also enter the chondrogenic differentiation pathway when cultured at super high density (micromass culture) and chondrogenesis is potentiated by growth factors such as BMP-2 or TGF-β1 [Denker et al., 1995, 1999]. In addition, a number of investigators have shown that components of the mitogen-activated protein kinase (MAP kinase) signaling pathway and their nuclear targets such as c-Fos, a component of activating protein-1 transcription factor (AP-1), are involved in the developmental process of chondrogenesis. Extracellular signal-regulated (ERK) and p38 MAP kinases have been reported to have opposing effects on the chondrogenic differentiation of micromass cultures of primary chick limb bud cells; inhibition of p38 results in reduced chondrogenesis whereas inhibition of MEK/ERK results in increased chondrogenesis [Oh et al., 2000]. Recent work also indicates that over expression of c-Fos, a target of the MAP kinase signaling pathway (MEK/ERK), inhibits chondrogenic activity of a mouse carcinoma derived chondrogenic cell line (ATDC5) in culture [Thomas et al., 2000]. Therefore, it appears that the ubiquitous MAP kinase signaling pathway and its nuclear targets play a pivotal role in regulating chondrogenesis.

Our recent work indicates that the binding and activity of the AP-1 transcription factor is markedly reduced shortly after C3H10T1/2 cells are plated as high density micromass cultures [Seghatoleslami and Tuan, 2002]. This change in AP-1 activity and binding is an early event which is maintained for at least 4 days during which C3H10T1/2 cells express chondrogenic genes and produce considerable amounts of negatively charged sulfated matrix components which can be stained with acidic alcian blue dye (pH 1). BMP-2 addition to micromass cultures of C3H10T1/2 cells induces chondrogenesis, but does not affect the activity of AP-1 significantly [Seghatoleslami and Tuan, 2002] suggesting that the long term maintenance of low AP-1 activity might play a necessary role in the overall molecular context of prechondrogenic cells which would favor the initiation and maintenance of chondrogenic gene expression. The long term effect of MAP kinase signaling, the signaling pathway regulating AP-1 activity, has also been compared to its short term effect on the behavior of PC12 cells (for review: Qui and Green, 1992; Marshall, 1995). Similar to our findings, this study also shows that the long term or persistent activity of ERK1 is important in neuronal differentiation of PC12 cells. In light of the above findings, our hypothesis is that MAP kinase activity, specifically extracellular signal-regulated kinase 1 and 2 (ERK1/2) activity that targets the components of nuclear factor AP-1, is differently regulated in chondrogenic and non-chondrogenic C3H10T1/2 culture conditions. Therefore, our aim in this work was to determine whether there is a difference in the duration and levels of ERK1/2 activity in the two culture conditions mentioned.

AP-1, comprising members of the Jun and Fos family of leucine zipper DNA binding factors, is one of the nuclear targets of the MAP kinase signaling pathway, which couples extracellular signals to changes in gene expression in the nucleus. Previously, we have shown that synthesis of c-Fos in C3H10T1/2 cells is dependent on cellular density; although cells which establish intercellular contacts up-regulate c-Fos, this activity peaks in super dense micromass cultures. For the studies presented in this article the transcriptional responsiveness of the *c-Fos* gene is used as a tool to study the complexity of the related signaling activities established by prechondrogenic condensing cells. The promoter elements of the *c-Fos* gene interact with number of transcription factors, which are targets for various cell signaling pathways.

The serum response element (SRE) interacts with a complex of nuclear factors that confer both growth factor- and stress-regulated activity on the *c-Fos* gene. Formation of this complex requires the initial binding of the ubiquitously expressed serum response factor (SRF) to the central element of SRE known as the CArG box [$CC(A/T)_6GG$], [Johansen and Prywes, 1995]. Upon binding as a homodimer, the SRF recruits accessory factors such as the Ets-family of ternary complex factors (TCFs) including ELK-1, Sap1, and Sap2/Net/Erp [Wasylyk et al., 1998]. Transcriptional activity is enhanced when TCFs are phosphorylated by various MAP kinases in response to growth factors and stress-inducing agents [Whitemarsh et al., 1995; Giovane et al., 1997; Clarke et al., 1998; Yang et al., 1998; Cruzalegui et al., 1999]. In addition to its obligate role in formation of SRE complex, SRF has also been shown to regulate transcription

in a TCF-independent manner, which is not well understood. In response to whole serum small GTP-binding proteins of the Rho family regulate c-Fos expression via SRF [Hill et al., 1995; Alberts et al., 1998]. It appears that SRF activity is targeted by several kinase cascades and, therefore, is a converging point for both growth factor-regulated and stress-activated signaling cascade as is the case for its interacting TCF partners. This induced TCF-independent activity of SRF is shown to be correlated with the phosphorylation of Ser-103 that is targeted by MAPKAP kinase 1 ($p90^{rsk}$) and 2, targets of both mitogen- and stress-activated MAP kinases [Heidenreich et al., 1999; Hanlon et al., 2001].

In this work, we have investigated the effect of cellular density, a condition influencing the initiation and progression of chondrogenesis, on the regulation of non-phosphorylated and phosphorylated levels of ERK1 and 2 in long term (2.5 day cultures) cultures of C3H10T1/2 cells. In addition, changes in the activity of ERK1/2 was assayed by measuring the activity of its nuclear targets, AP-1 and SRF. For functional analyses of AP-1 and SRF, we have used our previously developed pool of C3H10T1/2 stable cell lines harboring a luciferase gene under control of seven tandem repeats of AP1 [Seghatoleslami and Tuan, 2002], or five tandem repeats of SRF binding sites fused to a TATA box (these cell lines are designated as AP1-10T1/2 and SRF-10T1/2, respectively). ERK1/2 induced changes in AP-1 activity were correlated with changes in the levels of AP-1 components, c-Fos and c-Jun and its DNA binding. In the culture conditions tested in this report, the effect of the changes in the activity of ERK1/2, in the absence or presence of BMP-2, on the overall cell signaling involved in chondrogenic gene regulation was also examined.

MATERIALS AND METHODS

Cell Culture

Establishment and characterization of cell lines were described previously [Seghatoleslami and Tuan, 2002]. Stable cell lines derived from the C3H10T1/2 line designated AP1-10T1/2 and SRF-10T/12, harbor the AP-1 DNA vector containing a seven concatamer of AP1 consensus binding sites, and a five concatamer of SRF consensus binding sites, respectively, which are both followed by a basic TATA transcriptional unit fused to a luciferase marker gene (Stratagene, Cedar Creek, TX). Establishment of high density micromass cultures of AP1-10T1/2 and SRF-10T1/2 cells, luciferase and protein assays were described previously [Seghatoleslami and Tuan, 2002]. For MEK inhibition studies, PD 98059 was added to cultures at 5–50 μM concentrations at the initial time of plating. To study the chondrogenic effect of BMP-2 and its effect on *c-Fos* gene expression, the medium was supplemented with 100 ng/ml of this growth factor.

Luciferase Assay

To measure the luciferase activity, cell extracts were harvested and assayed as described elsewhere [Seghatoleslami and Tuan, 2002]. For micromass cultures, per 35-mm culture dishes, two droplets of 50 μl containing 5×10^5 cells each were plated. For the experiments shown in Figure 1 to establish low density monolayer cultures with minimum cell contact (40% confluent cultures), 5×10^5 cells were spread on 35-mm dishes. To establish confluent monolayer cultures with maximum cell contact, 35-mm culture dishes were plated at a cell density of 1×10^6 cells/dish. Unless otherwise mentioned, the monolayer cultures were established at 5×10^5 cells per 35-mm dish.

Preparation of Nuclear and Cytoplasmic Extracts

Nuclear extractions were performed as described previously [Seghatoleslami and Tuan, 2002]. Briefly, five to ten micromass cultures of C3H10T1/2 cells were used, each initially plated at 5×10^5 cells/50 μl medium. For monolayer cultures (low density monolayer cultures with minimized cell contact; 40% confluent culture), $1–2 \times 10^6$ cells/100-mm dish and 10 dishes per extraction were plated. Cultures were maintained for 2.5 days and then washed twice in cold phosphate buffered saline (PBS), scraped into 1 ml of PBS, and pelleted in microfuge tubes at low speed (6,000 rpm for 15 s). The pellets were then 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 μM β-glycerophosphate), containing 50 μl/ml protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma, St. Louis, MO), 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

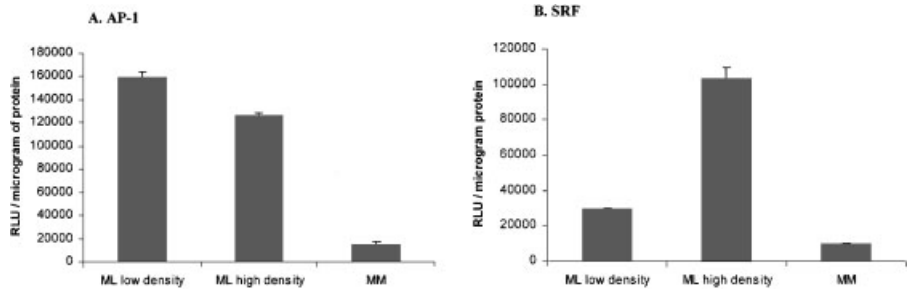


Fig. 1. The activity of activation protein-1 (AP-1) and serum response factor (SRF) transcription factors are affected by changes in C3H10T1/2 cell seeding density. The activity of AP-1 in AP1-10T1/2 (**A**) and SRF in SRF-10T1/2 (**B**) stable cell lines (establishment of AP1-10T1/2 and SRF-10T1/2 cell lines are described in Materials and Methods) were measured in 2.5 day cultures with different cell seeding densities. A cell density of 5×10^5 cells per 35 mm plate represents culture conditions in which cells make minimum contact (ML low density), while 1×10^6 cells 35-mm dish is the culture condition in which cells make maximum contact (ML high density). Micromass cultures (1×10^5 cells/10 μl) represent super-dense cultures, providing the appropriate cell–cell and cell–matrix contact, required for induction of chondrogenesis in C3H10T1/2 cells. Data is presented as average luciferase activities from duplicate experiments normalized to the protein content of the respective cultures.

dounce homogenizer. The nuclei were collected by centrifugation at 2,500 rpm 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 μM β-glycerophosphate) containing the same protease and phosphatase inhibitors as in buffer A, and incubated on ice for 90 min, followed by centrifugation at high speed (12,000 rpm) for 15 min. Aliquots of the supernatants containing the nuclear extract were frozen at −70°C until use. The aqueous fractions in buffer A from the first spin were used as the cytoplasmic fraction. The Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) was used to determine the protein content of both cytoplasmic and nuclear extracts.

Gel Shift Analysis

A double-stranded oligo deoxynucleotide (ODN) containing the AP1 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.

SDS–Polyacrylamide Gels (PAGE) and Western Blot Analysis

Equal amounts (4 μg) of the nuclear extracts used for gel shift assays were separated on 10% SDS–PAGE and electrophoretically transferred to nitrocellulose. Blots were blocked by incubation in Tris-buffered saline containing 3% bovine serum albumin and 0.05% Tween-20 and then subjected to rabbit polyclonal antibodies directed to the amino terminus of c-Fos (***SC-52***) and c-Jun (***SC-45***). Portions of cytoplasmic extracts obtained during the nuclear extraction preparation method described above were used to detect total and phosphorylated forms of ERK/12, by Western blot analysis. For ERK detection, 20 μg of total cellular extracts from subconfluent cultures containing non-contacting cells (5×10^5 cells/35-mm dish), confluent cultures (1×10^6 cells/35-mm dish), or micromass cultures (1×10^5 cells/10 μl) were separated on a 10% SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane. The blots were blocked as above and subjected to rabbit polyclonal antibodies against ERK1 and ERK2 (**K-23**; this antibody has weak interaction with ERK1) or to a mouse monoclonal antibody directed to phosphorylated form of ERK1/2 (p-ERK; **E-4**). Secondary antibody labeling was done using species specific HRP conjugated antibodies, followed by chemiluminescent detection using the Amersham ECL detection system (Amersham Biosciences, Piscataway, NJ). For these experiment, after detection of the c-Fos protein, the blot was stripped according to the ECL protocol provided by Amersham and reprobed with the c-Jun antibody as described above. Likewise, after detection of ERK proteins the blot was stripped and re-probed for pERK. Band intensities were normalized to the β-actin levels detected in each sample. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

RNA Extraction and Reverse Transcribed-Polymerase Chain Reaction (RT-PCR)

Ten micromass cultures per treatment, each containing 10×10^5 cells/100 μl micromass, were lysed and dissociated in 1 ml of TriZol reagent (Gibco BRL, Life Technologies, Grand Island, NY) and total RNA was isolated according to the manufacturer's protocol. Complementary DNA was reverse transcribed (RT) from 500 ng of total RNA using oligo dT primer and the Superscript™ First Strand Synthesis System for RT-PCR (Gibco BRL, Life Technologies) and specific amplification by PCR using PlatinumTaq DNA Polymerase enzyme (Gibco BRL, Life Technologies) was done using the following mouse-specific primer sets (forward/reverse): c-Fos, GAGCTGACAGATACACTCCAAGCG/CAGTCTGCTGCATAGAAGGAACCG; Sox9, TCCTAGTCTAGACACGCTCGCGTG/GCTCTCGGCTCTCCGACTTCC; aggrecan, TTGCCAGGGGGAGTTGTATTC/GACAGTTCTCACGCCAGGTTTG; type II collagen, GTGAGCCATGATCCGC/GACCAGGATTTCCAGG. PCR band intensities were normalized to the expression of the house-keeping gene, GAPDH.

RESULTS

Effect of High Density Micromass Cultures on AP-1 and SRF Activity

Previously, we have shown that an increase in C3H10T1/2 cell density causes a large drop in AP-1 activity. Despite lower AP-1 binding and activity in these dense micromass cultures, a culture condition necessary for initial cellular condensation and initiation of chondrogenesis, induction of c-Fos protein, a component of the AP-1 heterodimer, was observed. As an indirect approach to determine whether this change in AP-1 activity was partly due to changes in transcriptional activity of c-Fos and also to identify other nuclear targets relevant to cell density dependent MAP kinase signaling, we have chosen to study the activity of SRF, an obligate component of a transcriptional complex interacting with the SRE DNA sequence present in the c-Fos promoter. Figure 1 shows the activity of AP-1 and SRF in AP1-10T1/2 and SRF-10T1/2 cells cultured at different cell densities. As reported previously, AP-1 activity is lower in cultures showing cell–cell contact and is markedly reduced in extremely dense micromass culture (Fig. 1A). Analysis of SRF-10T1/2 cells also indicates that activity of the SRF transcription factor is decreased when cells are plated at high micromass density (Fig. 1B), whereas unlike AP-1, the activity of SRF in confluent cultures of C3H1T1/2 is up-regulated suggesting a difference in the molecular activity of confluent (in comparison with low density monolayer cultures in which cellular contact is minimized) and super dense micromass cultures (Fig. 1A,B).

Micromass Cell Seeding Density of C3H10T1/2 Cells Results in Induction of c-Fos as Well as Genes Specific to Chondrogenesis

Since c-Fos overexpression is shown to influence chondrogenesis [Thomas et al., 2000], we assessed the chondrocyte specific gene regulation in relation to the expression of *c-Fos* gene. As shown in Figure 2, RT-PCR analysis indicates that expression of the c-Fos message is up-regulated upon C3H10T1/2 cell contact; activity of c-Fos peaks when C3H10T1/2 cells are either cultured as micromass (Fig. 2, lanes 1 and 6) or as a low density monolayer making increasing cell–cell contact with time (Fig. 2, compare 2.5 and 4.5 day monolayer cultures; lanes 5 and 10). While monolayer cultures express type IIA collagen, a spliced form of type II collagen associated with pre-chondrogenic cells [Nah and Upholt, 1991], expression of type IIB collagen, a spliced form of type II collagen associated with differentiating chondrocytes, is up-regulated in cells making increasing contact or when C3H10T1/2 cells are cultured in micromass densities (Fig. 2; lanes 1, 5, and 10). Increase in the expression of Sox9 encoding an important transcription factor in regulating type II collagen gene and chondrogenesis is also associated with increased levels of type II collagen mRNA (Fig. 2). Aggrecan, a chondrocyte specific proteoglycan, is not expressed during early stages of C3H10T1/2 chondrogenesis (Fig. 2, lanes 1 and 6; 2.5–4.5 day micromass cultures). Aggrecan gene expression was induced when chondrogenic activity of micromass cultures was induced with BMP-2 (Fig. 2, lanes 3 and 8). These observations, therefore, suggest that cell density dependent upregulation of *c-Fos* gene occurs in the context of chondrocyte specific signaling activities. Treatment of micromass cultures of C3H10T1/2 cells with BMP-2 (100 ng/ml) resulted in a more advanced stage of chondrogenesis than untreated cultures evidenced

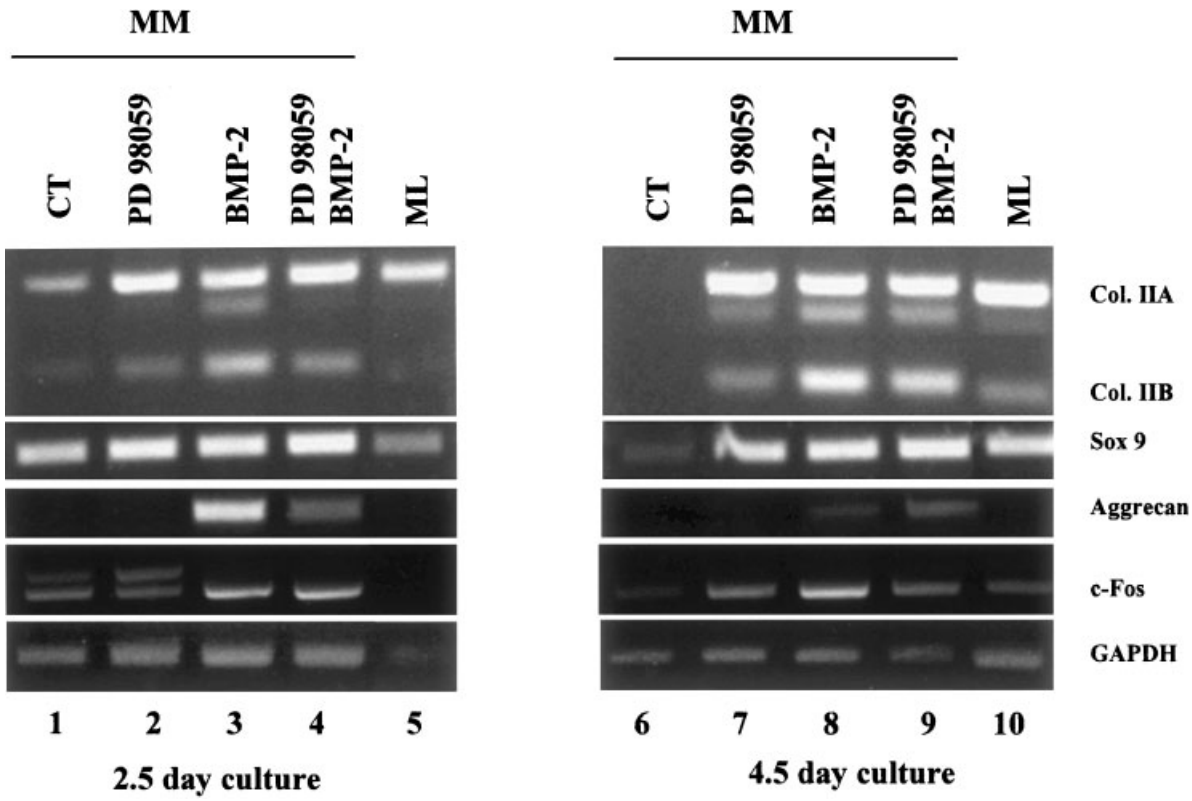


Fig. 2. Effect of cell density on the response of *c-Fos* and chondrogenic genes to the changes in extracellular signal-regulated kinase (ERK) and BMP-2 signaling. The activity of endogenous *c-Fos* and chondrogenic genes type II collagen, aggrecan and Sox9 were measured in C3H10T1/2 cells cultured either as monolayer or micromass. Activities were measured using reverse transcriptase-PCR. Untreated monolayer cultures (ML, **lanes 5** and **10**) and untreated micromass cultures (MM; CT, **lanes 1** and **6**) and treated with 5 μM PD 98059 (**lane 2** and **7**), 100 ng/ml BMP-2 (**lane 3** and **8**), PD 98059 and BMP-2 (**lane 4** and **9**), are shown in this figure. Cells were cultured for either 2.5 days (**left panel**) or 4.5 days (**right panel**).

by increase in mRNA levels for type IIB collagen, aggrecan and c-Fos (Fig. 2, compare lanes 1 and 3). As compared with 2.5 day micromass cultures, BMP-2 treatment of similar cultures for longer period of time (4.5 days) resulted in fourfold reduction in aggrecan gene expression (Fig. 2, compare lanes 3 and 8). This reduction in aggrecan gene expression in 4.5-day micromass cultures is concomitant with a 2.2-fold increase in c-Fos mRNA levels as compared with 2.5-day cultures.

Effect of PD 98059 Inhibition on c-Fos and Chondrogenic Gene Activity in Micromass Cultures of C3H10T1/2 Cells

Next we examined the effect of PD 98059, a specific inhibitor of MEK/ERK, on activity of c-Fos, a known target of ERK1/2 mitogen activated kinases, and the activity of the chondrocyte specific genes as analyzed above. In comparison with control cultures, inhibition of MEK/ERK in 2.5 day micromass cultures of C3H10T1/2 cells with 5 μM PD 98059 resulted in a reduction in c-Fos levels (Fig. 2, compare lanes 1 and 2; 26% reduction) while in the BMP-2 treated cultures inhibition of ERK1/2 did not change the activity of this gene (Fig. 2, compare lanes 3 and 4). In 4.5 day cultures, on the other hand, ERK inhibition did not confer the same effect on *c-Fos* gene activity; while ERK inhibition resulted in induced activity of *c-Fos* gene in untreated cultures (Fig. 2, compare lanes 6 and 7; 49% induction) the activity of this gene was inhibited in BMP-2 treated cultures (Fig. 2, lanes 8 and 9; more than 16% inhibition). PD 98059 inhibition of both 2.5 and 4.5 day micromass cultures resulted in induced activity of type II collagen gene (Fig. 2, compare lanes 1 and 2, lanes 6 and 7). PD 98059 inhibition of both 2.5 and 4.5 day BMP-2 treated micromass cultures on the other hand resulted in reduced activity of type IIB collagen gene (Fig. 2, compare lanes 3 and 4, lanes 8 and 9). We have also observed a time dependent effect of PD 98059 on the

activity of the aggrecan gene in micromass cultures of C3H10T1/2 cells; whereas ERK inhibition of 2.5 day cultures treated with BMP-2 resulted in down regulation of aggrecan mRNA (Fig. 2, compare lanes 3 and 4; more than twofold reduction) the activity of this gene was up-regulated in similar 4.5 day cultures (Fig. 2, compare lanes 8 and 9; threefold induction) which was associated with reduced c-Fos mRNA levels (more than 16% decrease). In general, *Sox9* gene expression, a chondrogenic specific transcription factor enhancing the activity of type II collagen gene, followed the similar changes in type II collagen mRNA levels induced by the treatments and culture conditions mentioned above (Fig. 2).

Inhibition of MEK Results in Delayed Induction of AP-1 and SRF Activity

Next we investigated the effect of ERK inhibition on the activity of AP-1, since c-Fos expression, a major component of AP-1 heteroduplex, is the target of the ERK MAP kinase. Figure 3A shows the effect of 5 μM PD 98059, a specific inhibitor of MEK1/2:ERK1/2, on the activity of AP-1 in AP1-10T1/2 cells at various times in micromass cultures. Following ERK inhibition, a significant but surprising increase in AP-1 activity was observed, beginning 5 h after cultures were established. In time the activity of AP-1 was further increased reaching a maximum level (more than twofold) within 24–48 h of ERK inhibition (Fig. 3A, 48 h activity is not shown). To test whether this increase in AP-1 activity is due to c-Fos transcriptional activity we determined the levels of c-Fos mRNA as well as the activity of SRF, an important transcription factor involved in MAP kinase regulation of *c-Fos* gene. As shown in Figure 3B, PD 98059 inhibition of SRF-10T1/2 cells in micromass cultures resulted in a gradual increase in SRF activity starting 5 h after treatment and reaching its peak of activity (close to twofold increase in activity) within 48 h. This pattern of SRF activity closely correlated with that of AP-1 in the AP1-10T1/2 cells inhibited with PD 98059 (Fig. 3A,B, respectively). Surprisingly, while there was a gradual rise in SRF activity in 2.5 day micromass cultures of C3H10T1/2 cells inhibited with 5 μM PD 98059, the transcriptional activity of the SRF-dependent *c-Fos* gene in the same culture condition was reduced (compare Figs. 3B and 2, lanes 1 and 2). On the other hand, MEK/ERK inhibition of similar

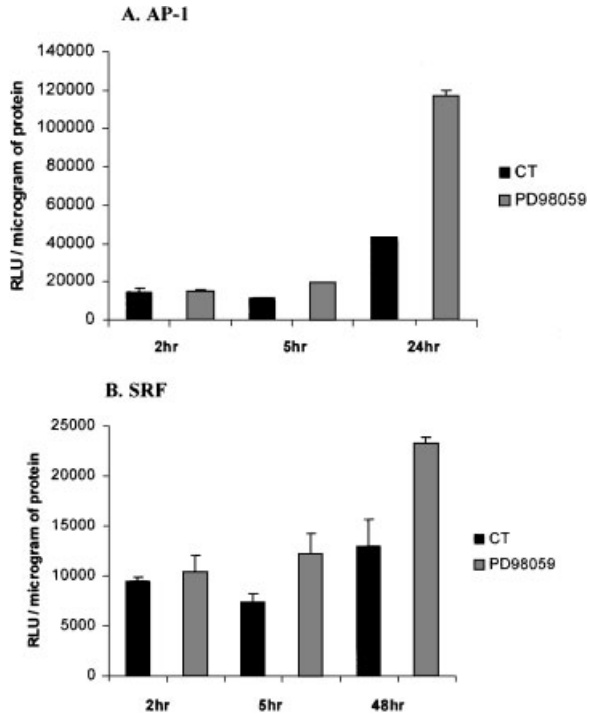


Fig. 3. MEK inhibition results in delayed induction of AP-1 and SRF. Luciferase activities obtained from micromass cultures of AP1-10T1/2 (**A**) or SRF-10T1/2 (**B**) cells that were untreated (CT) or treated with 5 μM PD 98059 inhibitor for various times in culture. Luciferase activities are normalized to protein content of each sample. Data indicates the average luciferase activity of duplicate cultures.

cultures for longer period of time (4.5 days) resulted in induced levels of c-Fos mRNA which is consistent with the induced levels of SRF activity (Figs. 3B and 2, lanes 6 and 7).

Concentration Dependent Effect of MEK Inhibitor on AP-1 and SRF Activity

Since the activity of AP-1 and SRF were induced in micromass cultures inhibited with 5 μM PD 98059 (Fig. 3), we were interested to know if inhibition of MEK/ERK with increasing concentrations of PD 98059 could restore the high monolayer activity of these transcription factors (Fig. 1A,B). For these experiments, micromass cultures were harvested 2.5 days after plating, the time point at which low concentrations of inhibitor (5 μM) resulted in the highest activity of SRF and AP-1 (Fig. 3). Treatment of AP1- and SRF-10T1/2 cells with 5–10 μM PD 98059 resulted in only two- to threefold increase in AP-1 or SRF activity in both micromass (Fig. 4A,C) as well as monolayer cultures (Fig. 4B,D). Treatment of these cultures with higher than 10 μM concentrations

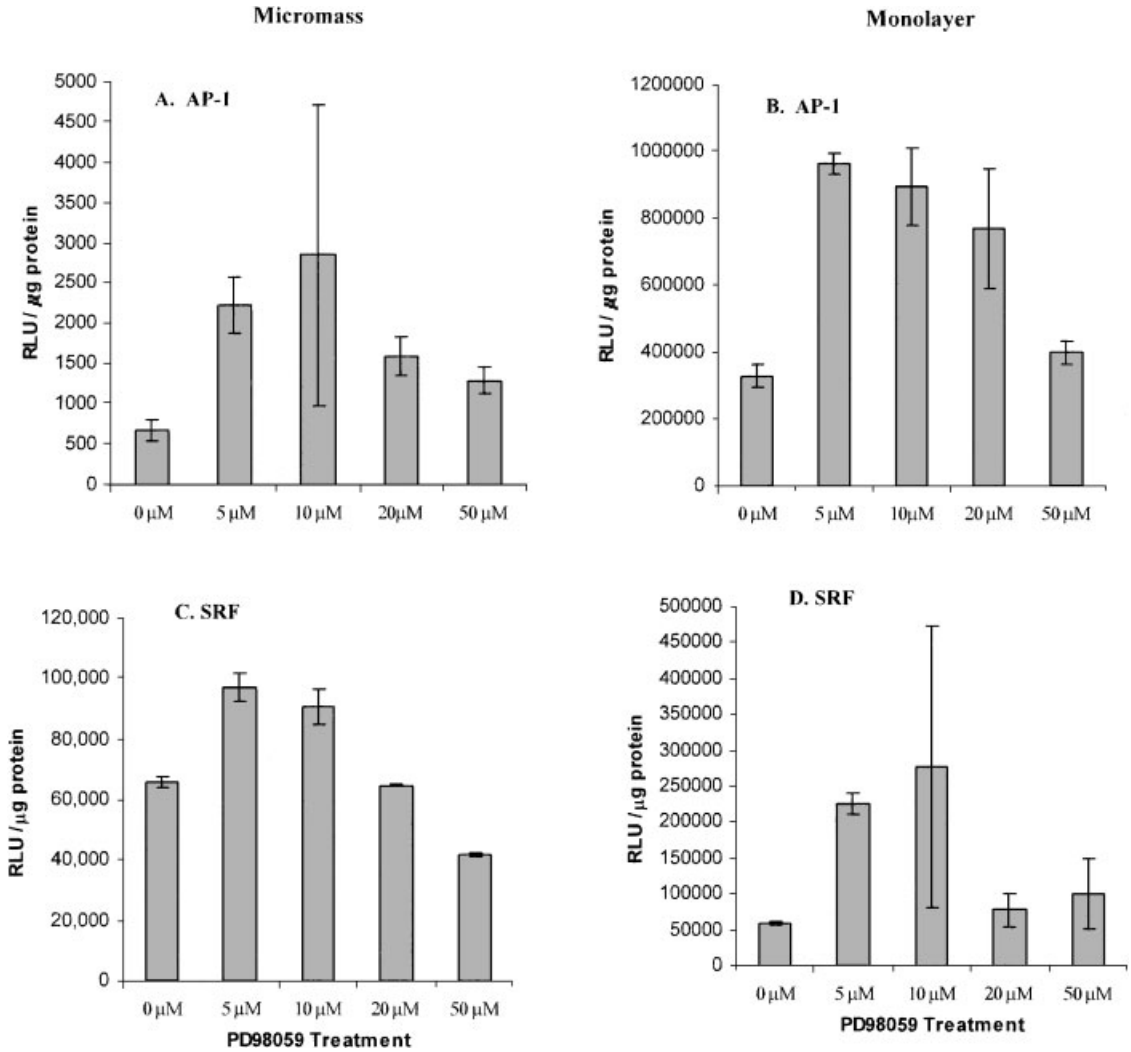


Fig. 4. Concentration dependent effect of PD 98059 on AP-1 and SRF activity. Micromass (**A** and **C**) and monolayer (**B** and **D**) cultures of AP1-10T1/2 or SRF-10T1/2 cells, respectively, were untreated (0 µM) or treated with different concentrations of PD 98059 inhibitor for 2.5 days, a time point at which maximum effect of ERK inhibition was observed. Luciferase activities were normalized to protein content of each sample. Data indicates the average luciferase activity of duplicate cultures.

of PD 98059 resulted in inhibition rather than further induction of AP-1 and SRF activities. PD 98059 treatment of the cultures at concentrations higher than 20 µM lowered the activity of SRF and AP-1 close to the levels observed for the non-treated cultures (Fig. 4A–D).

Using SDS–PAGE, we analyzed the effect of cell density and the PD 98059 inhibitor on synthesis of the unmodified and activated phosphorylated forms of ERK1/2 (pERK1/2). As shown in Figure 5B, in long term monolayer cultures (2.5 day) there is a correlation between the concentration of inhibitor used and the total as well as activated levels of ERK1/2; higher the amount of inhibitor used lower the total levels of ERK1/2 (the antibody used has a weak interaction with ERK1) or pERK1/2 seen in monolayer cultures (Fig. 5B). In the monolayer cultures of C3H10T1/2 cells the ERK1 and 2 differ in their response to PD 98059 inhibitor which is consistent with the findings of Alessi et al.; the levels of ERK1 and its phosphorylated form pERK1 are down-regulated at lower inhibitor concentrations than that of ERK2 and pERK2 (Fig. 5B). The total levels and activity of ERK1/2 in micromass cultures, however, are less affected by PD 98059 inhibition (Fig. 5A). Despite a sharp drop in the activity of AP-1 and SRF in micromass cultures the levels of their affector kinases ERK1/2 were not as sharply affected

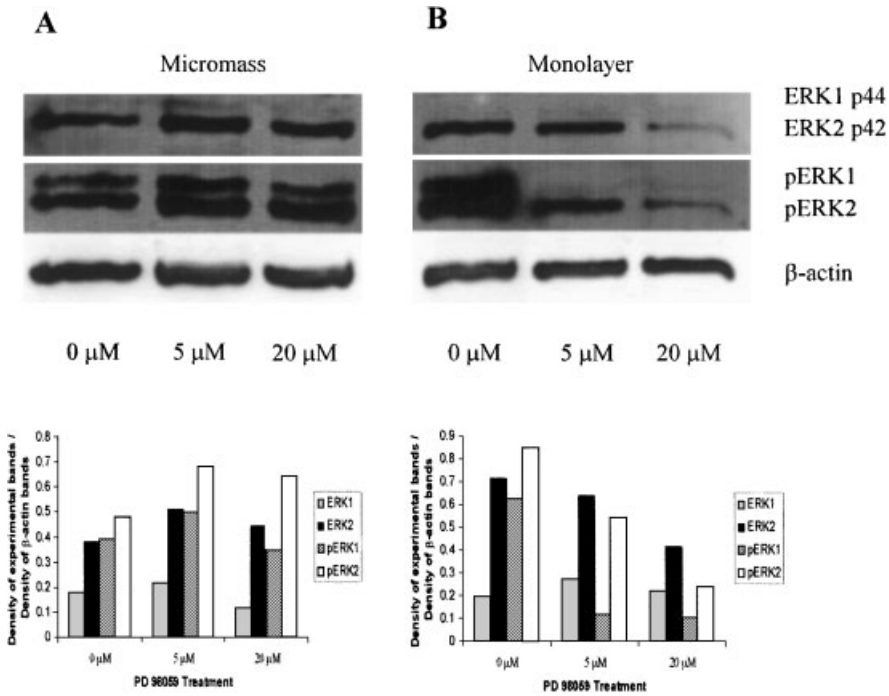


Fig. 5. Activity and levels of ERK1/2 in monolayer or micromass cultures of C3H10T1/2 cells treated with increasing concentrations of PD 98059 inhibitor. SDS–polyacrylamide gels (PAGE) was used to fractionate cytoplasmic protein extracts isolated from micromass (**A**) or monolayer cultures (**B**). The protein bands were electrophoretically transferred to a nitrocellulose membrane and probed with polyclonal antibodies detecting ERK2 or pERK1/2 (Materials and Methods). The reactive proteins were visualized by ECL Western blotting detection system (Amersham Biosciences, Piscataway, NJ) as described in Materials and Methods. Each panel of antibody labeling is from the same blot. Using stripping protocol described by the manufacturer (Amersham Biosciences), the same blot was used for multiple antibody detections. The charts shown at the bottom of each panel indicate the comparison of the experimental band densities normalized to the respective β-actin band densities from each sample. Density of bands were determined using *Kodak Digital Science* 1D Image Analysis Software.

and in the inhibited cultures the levels and activity of these kinases were enhanced rather than lowered (compare Figs. 1 and 5).

Since the components of AP-1 transcription factor are subject to phosphorylation that changes their DNA binding ability we decided to examine the binding of AP-1 to its consensus sequence using retarded gel mobility shift assay. This analysis indicated that there is a direct correlation between the activity of AP-1 and its DNA binding ability in micromass cultures; use of the inhibitor at 5–10 μM resulted in an increase of AP-1 binding and activity while at 20–50 μM a reduction was observed (Fig. 6; compare lanes 4, 5, and 6). Interestingly, despite a large increase in DNA binding (Fig. 6; compare lanes 4 and 5) the activity of AP-1 in the PD 98059 inhibited micromass cultures was not restored to the level of AP-1 activity observed for the untreated monolayer cultures (Figs. 1 and 6; compare the binding and activity of AP-1, respectively). Whereas the pattern of the activity of AP-1 in PD 98059 inhibited monolayer cultures of AP1-10T1/2 paralleled that of the micromass cultures, its DNA binding activity is opposite to what is observed for the micromass cultures; increasing concentrations of inhibitor resulted in reduced DNA binding of AP-1 in monolayer cultures (Fig. 6; compare lanes 1, 2, and 3).

SDS–PAGE analysis of the same nuclear extracts used in the gel shifts also indicates that the effect of PD 98059 inhibitor on the expression of major components of AP-1, c-Fos and c-Jun, is cell density dependent. As Figure 7A indicates, both c-Jun and c-Fos protein levels are up-regulated when micromass cultures are inhibited with increasing amounts of PD 98059 for 2.5 days; use of this inhibitor at higher concentrations (more than 5 μM) increases the c-Jun levels while that of c-Fos remains unaffected. On the other hand, 2.5-day inhibition of ERK1/2 in monolayer cultures results in a different pattern of *c-Jun* and *c-Fos* gene activity.

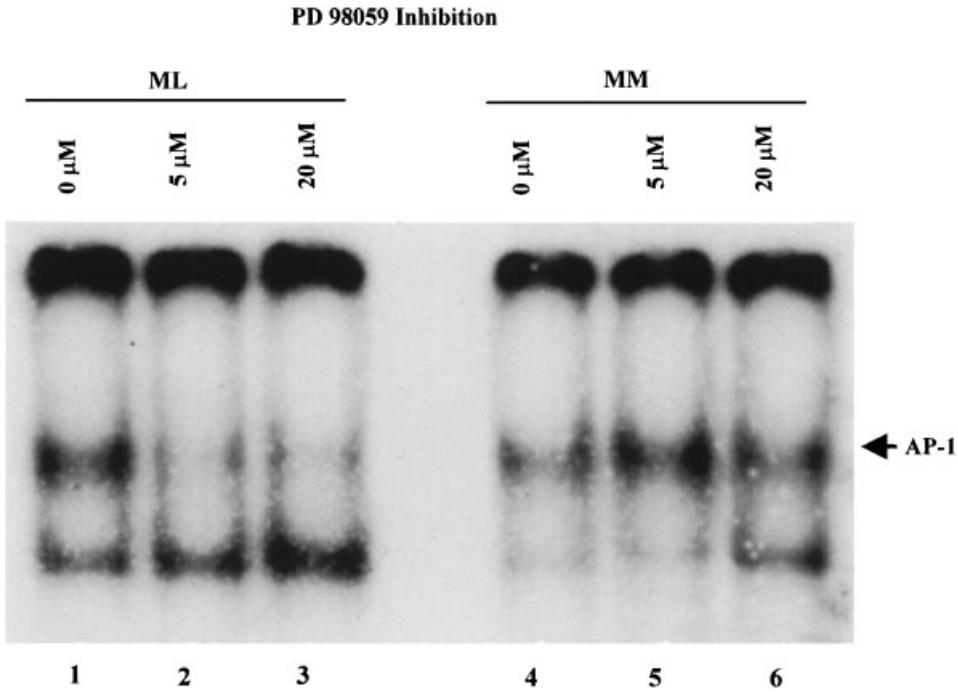


Fig. 6. Effect of increasing concentrations of PD 98059 inhibitor on the DNA binding ability of AP-1. The same extracts shown in Figure 7 were used in AP-1 mobility gel shift DNA binding assay. The sequence of AP-1 oligo and the methodology used for mobility gel shift analysis are described in Material and Methods. **Left panel** shows the pattern of AP-1 binding in the nuclear fraction of 2.5 day monolayer (ML) cultures untreated (0 μM) or treated with different concentrations of PD 98059. The **right panel** shows the pattern of AP-1 binding in the nuclear fraction isolated from 2.5 day micromass cultures (MM) treated in the same manner as for the monolayer cultures.

Whereas the levels of c-Jun in monolayer cultures treated with various concentrations of PD 98059 are relatively unaffected, the levels of c-Fos, contrary to its levels in micromass cultures, are progressively reduced (Fig. 7B). Therefore, in micromass as well as monolayer cultures of C3H10T1/2 cells inhibited with PD 98059, we can draw a direct correlation between the DNA binding capacity of AP-1 and the levels of c-Fos protein available. Taken together the above data suggest that change in cellular density in C3H10T1/2 cultures differentially regulates the level and activation of ERK1/2. In addition, the above findings also indicate that there is not a close correlation between the binding and activity of AP-1 whose components are known targets of ERK1/2.

DISCUSSION

Disruption of cellular condensation, an early required stage of chondrogenesis, results in reduced chondrogenic specific gene activity [Oberlender and Tuan, 1994a,b; Seghatoleslami and Kosher, 1996]. Previously, we have shown that maintenance of low and steady levels of the AP-1 transcription factor [Seghatoleslami and Tuan, 2002], a nuclear target of ubiquitously active MAP kinase signaling activity, is an early molecular event initiated by cellular condensation during chondrogenesis. The transcription factor, AP-1, is a dimer composed of different protein subunits of the Jun and Fos family among which the c-Fos/c-Jun heterodimer binds DNA at a higher affinity and confers positive transcriptional activity [Halazonetis et al., 1988; Kouzarides and Ziff, 1988]. More interestingly, in addition to this DNA-binding dependent activity (for review see Karin et al., 1997), AP-1 can exhibit DNA-binding independent activity that involves protein–protein interaction with other transcription factors as well as nuclear co-factors (for reviews see Karin, 1998; Herrlich, 2001; Karin and Chang, 2001). This dual function of the AP-1 transcription factor, therefore, allows modulation of different AP-1 as well as non-AP-1 targeting cell signaling activities, which in turn affect multiple cellular functions [Johnson et al., 1992; Hilberg et al., 1993].

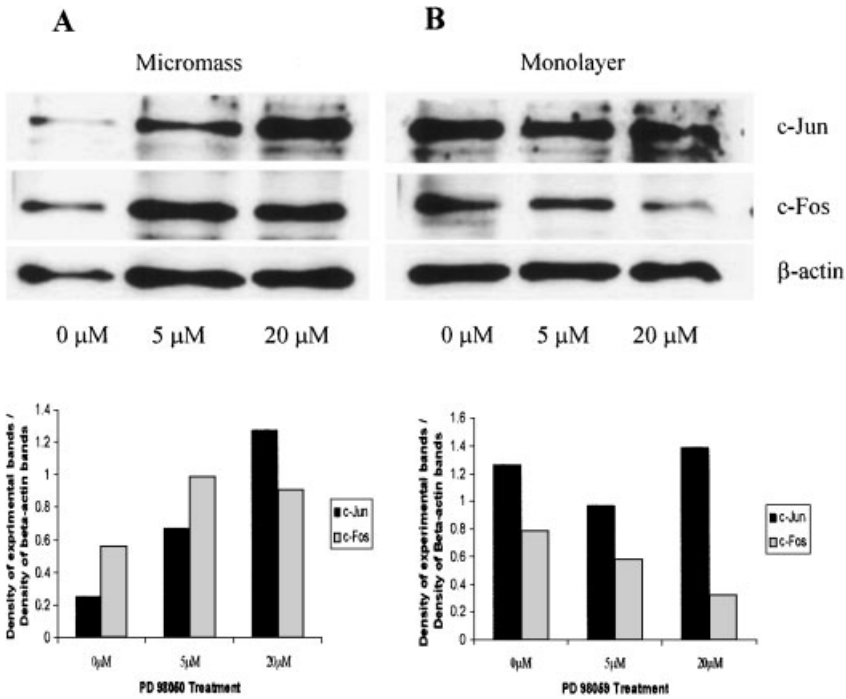


Fig. 7. Effect of increasing concentrations of PD 98059 inhibitor on the levels of c-Fos and c-Jun in chondrogenic or non-chondrogenic C3H10T1/2 cells. Nuclear fractions (4 μg) from micromass (**A**) or monolayer (**B**) cultures of C3H10T1/2 cells untreated (0 μM) or treated with PD98059 inhibitor were fractionated using 10% SDS–PAGE. Antibodies used are described in Materials and Methods. **Lower panel**: c-Fos, 46 kDa; **upper panel**: c-Jun, 34 kDa. The same blot for each panel was stripped and probed for different proteins as described for Figure 5. The charts shown under each panel are the comparison of the experimental band densities normalized to the respective β-actin band densities from each sample. Density of bands were determined using *Kodak Digital Science* 1D Image Analysis Software.

Chondrogenesis is affected by the activity of the components of MAP kinase signaling cascade as well as that of AP-1. For example, over expression of c-Fos is associated with inhibition of chondrogenesis in ATDC5 cells [Thomas et al., 2000] and components of the MAP kinase signaling pathway, i.e., p38 and ERK, the signaling pathway affecting AP-1 binding and activity, have been shown to confer opposing effects on chondrogenesis of primary chick limb mesenchymal cells in culture [Oh et al., 2000]. Therefore, to understand the complexity of immediate changes in the molecular activity of C3H10T1/2 cells undergoing chondrogenesis, we have investigated the effect of cell condensation on the levels and activity of components of the AP-1 nuclear factor. In addition, we have investigated the activity of SRF, a transcriptional regulator of the *c-Fos* gene. AP-1 and SRF are considered immediate early genes, in relation to their interacting MAP kinase signaling pathway. In addition to the work of other investigators, two major observations have been made in our previous work [Seghatoleslami and Tuan, 2002] that led us to study the complexity of the ubiquitously active MAP kinase signaling in chondrogenesis: first, the activity of AP-1, the nuclear target of MAP kinase, is maintained steadily low and this is consistent with the reported requirement that long term maintenance of MAP kinase activity is necessary to induce cellular differentiation [Qui and Green, 1992]. Secondly, although there was a large increase in levels of c-Fos in micromass cultures of C3H10T1/2 cells, the binding and activity of AP-1 was maintained low. These findings suggest that there is a cell density dependent change in ubiquitous signaling activities, as evidenced by the pattern of AP-1 and SRF activities in monolayer and micromass cultures of C3H10T/12 cells, which could potentially result in differential gene regulation. In addition, the pattern of activity of SRF and AP-1 in confluent cultures of C3H10T1/2 cells indicates that changes in cell–cell interaction confer distinct effects on the activity of different branches of the MAP kinase pathway.

Upon examination of the activity of the *c-Fos* gene, an SRF responsive gene, using RT-PCR analysis, we detected a rise in c-Fos transcript

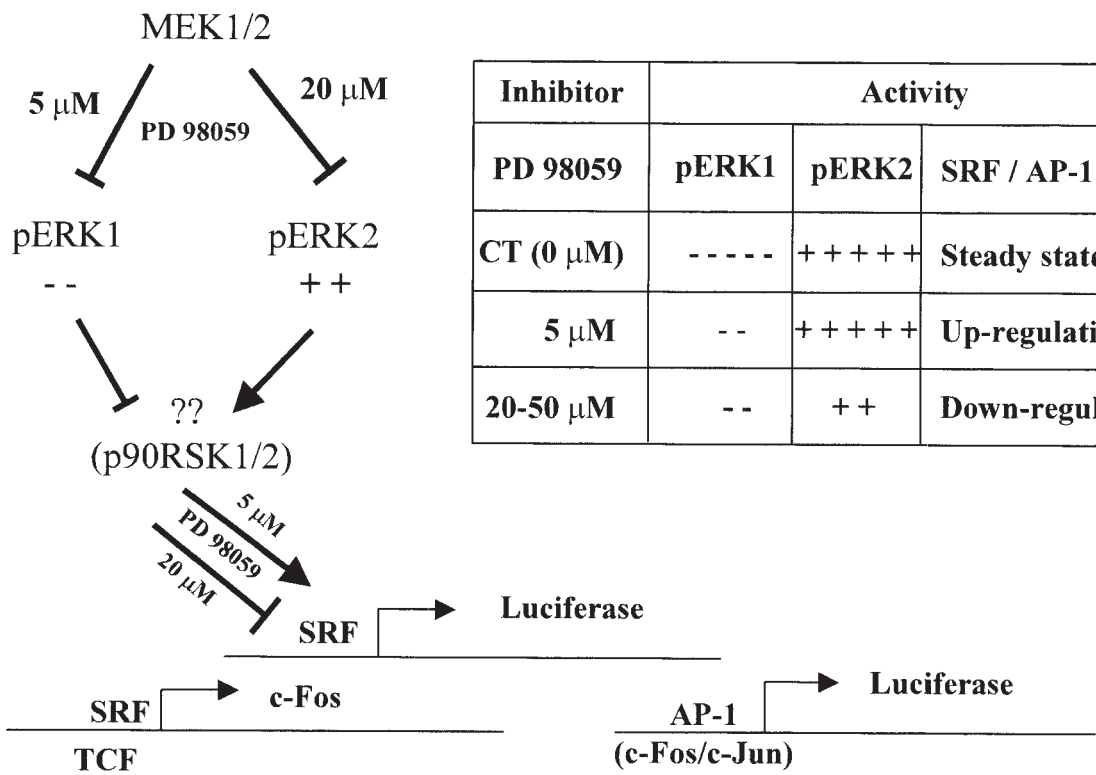
as the cells in culture made increasing contact. Whereas the activity of *c-Fos* gene in sub-confluent (reduced contact) and confluent monolayer cultures is consistent with the activity of the SRF transcription factor in similar cultures, this correlation can not be made in micromass cultures in which activity of SRF drops while that of c-Fos is greatly enhanced. Therefore, this observation suggests that the mechanisms involved in transcription of the *c-Fos* gene might be less dependent on SRF function in micromass than it is in monolayer cultures.

We further investigated the effect of the compound PD 98059, a specific inhibitor of the MAP kinases, MEK/ERK that negatively regulate chondrogenesis [Oh et al., 2000], on AP-1 and SRF activity. Surprisingly, inhibition of ERK1/2, known to affect the activity of c-fos, did not cause an immediate change in the activity of AP-1 in micromass cultures of AP1-10T1/2 cells, rather through a delayed mechanism this activity, instead of being inhibited, was induced, starting after 5 h of culture and reaching a maximum activity with a twofold increase within 48 h. This pattern of activation was mimicked by the transcription factor, SRF, suggesting that both of these transcription factors are long term targets of ERK activity in micromass cultures of C3H10T1/2 cells. From previous studies on the c-Fos promoter [Shaw et al., 1989; Hill et al., 1993; Johansen and Prywes, 1993] it is known that SRF binding to the SRE element is necessary for the formation of a complex involving TCFs. This complex engages the activity of several signaling pathways that regulate the induction of the *c-Fos* gene [Treisman, 1992]. These transcription factors involved in regulating the transcription of *c-Fos* gene, the TCFs and SRF, are known immediate targets of ERK1/2 [Treisman, 1994; Whitemarsh et al., 1995; Price et al., 1996]. The delayed increase in SRF or AP-1 activity in response to a low concentration of PD 98059 suggests that in chondrogenic C3H10T1/2 cultures (micromass cultures), despite an increase in expression and phosphorylation of ERK1/2 (Fig. 5), the effect of ERK1 and 2 on the activity of these transcription factors is tightly controlled and possibly involves the interaction of MAP kinase signaling with that of other signaling pathways; cell signaling composition of micromass cultures does not allow immediate changes in AP-1 and SRF activity that can be targeted by ERK1/2. On the other hand, SRF activity can also be targeted indirectly by ERK activated $p90^{RSK}$ [Rivera et al., 1993; Hanlon et al., 2001; Heidenreich et al., 1999] and whether the complexity of this mode of ERK1/2 action can account for the delayed response of SRF is the subject of further investigation. Treatment of monolayer or micromass cultures of C3H10T1/2 cells with higher concentrations of PD 98059 inhibitor (10–50 μM) results in decreased activity of AP-1 as well as SRF, reaching the levels of activities seen for untreated cultures (Fig. 4).

The drop in activity of AP-1 and SRF in micromass cultures and the differential activity of these nuclear factors in response to increasing levels of PD 98059 prompted us to compare the levels of ERK1 and 2 and their phosphorylated forms in micromass and monolayer culture systems. Recent studies by Oh et al. indicate that inhibition of ERK1/2 with a low concentration of PD 98059 (5 μM) resulted in an induction of chondrogenesis in micromass cultures of early chick limb bud mesenchymal cells. Our results indicate that in C3H10T1/2 mouse embryonic cells, inhibition of different forms of ERK1/2 by PD98059 is dependent on cell density. Long term (2.5 day) treatment of monolayer cultures with increasing concentrations of this inhibitor resulted in reduced levels of total as well as phosphorylated forms of ERK1/2, whereas the same treatment in micromass cultures resulted in an opposite effect (Fig. 5). Therefore, long term (2.5 day) treatment of C3H10T1/2 cells with PD 98059 results in autoregulation of ERK1/2 total levels which is cell density dependent. In both culture systems, however, the activity of AP-1 and SRF, whether controlled by ERK1/2 or other substituting signaling events, did not change significantly which supports our hypothesis that a maintained threshold level of activity for these transcription factors in pluripotent C3H10T1/2 cells is important during chondrogenesis. These findings again suggest that cell density dependent signaling activities that control the function of AP-1 and SRF are complex and possibly involve the cross interaction of other signaling pathways with that of MAP kinase.

Specific phosphorylation (activity) of MEK1 and 2 is shown to be affected by different concentrations of PD 98059 inhibitor [Alessi et al., 1995]; a low concentration (5 μM) of PD 98059 inhibitor is associated with inhibition of MEK1/ERK1 phosphorylation whereas MEK2/ERK2

phosphorylation is affected at higher concentrations of this inhibitor (20–50 μM). In agreement with Alessi et al. our data presented in Figure 5, particularly for monolayer cultures, also indicate that the phosphorylation of ERK1 and 2 in C3H10T1/2 cells is dependent on the concentration of PD 98059 used. Therefore, we suggest that ERK1 and 2 have opposing effects on the activity of AP-1 and SRF; ERK1 can negatively affect AP-1 and SRF while ERK2 functions as a positive regulator. Furthermore, to maintain a threshold of AP-1 and SRF activity, as shown in Figure 8, we propose that pluripotent C3H10T1/2 cells maintain finite but equal levels of ERK1/2 activity. In such a signaling mechanism, low concentrations of PD 98059 inhibit ERK1 phosphorylation which leads to dominance of ERK2 dependent up-regulation of AP-1 and SRF while at higher concentrations ERK2 activity is also inhibited leading to a subsequent reduction in the activities of AP-1 and SRF. Therefore, as our findings indicate, a tightly controlled and balanced activity of ERK1 and ERK2 maintains steady levels of AP-1 and SRF nuclear activities, which could play a significant role in initiation and maintenance of chondrogenesis in C3H10T1/2 cells. Recent studies suggest that SRF can regulate the function of genes involved in proliferation and differentiation. In addition, this transcription factor can serve as an immediate target of caspase activity thereby inducing apoptosis [Gauthier-Rouviere et al., 1996; Soulez et al., 1996; Arsenien et al., 1998; Bertolotto et al., 2000; Drewett et al., 2001; Kim et al., 2001]. Furthermore, this transcription factor, as a converging point of different cell signaling events including that of different



Inhibitor	Activity		
PD 98059	pERK1	pERK2	SRF / AP-1
CT (0 μM)	- - - - -	+ + + + +	Steady state
5 μM	- -	+ + + + +	Up-regulation
20-50 μM	- -	+ +	Down-regulation

Fig. 8. Possible scheme for delayed effect of PD 98059 inhibitor on the activity of AP-1 and SRF. Long term differential effect of ERK1/2 on AP-1 and SRF activity. This schematic description of AP-1 and SRF activities is based on the assumption that there is an equal but finite levels of ERK1 and 2 activities present in micromass cultures of C3H10T1/2 cells. Treatment of C3H10T1/2 cells with low concentration (5 μM) of PD 98059 (MEK inhibitor) results in inhibition of ERK1 activity which in turn leads to up-regulation of SRF or AP-1 activity; ERK1 functions as a negative regulator of AP-1 or SRF activity. Higher concentrations (10–50 μM) of MEK inhibitor in addition to ERK1 will also prevent the phosphorylation of ERK2 leading to down-regulation of the AP-1 and SRF activities; ERK2 functions as an activator of these transcription factors. This delayed effect of ERK1/2 in micromass cultures of C3H10T1/2 cells could be due to a secondary effect involving differential regulation of p90RSK1/2 or other unknown signaling events. SRF activity affecting c-Fos transcription is one of the nuclear targets for p90RSK1/2. Transcriptional effect of SRF on *c-Fos* gene in turn will determine the level of AP-1 activity.

branches of MAP kinase pathway, regulates the activity of the *c-Fos* gene which is known to have a major role during chondrogenesis of ATDC5 cells [Thomas et al., 2000]. Therefore, SRF like c-Fos, a component of the AP-1 heterodimer, and as a target of MAP kinase signaling, could contribute to the molecular activities involved in the early stage of chondrogenesis. So far, our findings suggest that during chondrogenesis of C3H10T1/2 cells in micromass cultures the maintained activity of AP-1 and SRF is tightly regulated by showing that inhibition of MEK1/2/ERK1/2 do not result in deviation of the activity of these transcription factors beyond a certain threshold. Our findings are also in line with the findings of Qui and Green, showing the importance of maintained MAP kinase activity in promoting cellular differentiation.

We extended our work further to determine the relationship between the pattern of binding and activity of AP-1 in the PD 98059 treated cultures. We have shown previously [Seghatoleslami and Rocky, 2002] and in the work reported here that there is a correlation between AP-1 activity and DNA binding in monolayer and micromass cultures of C3H10T1/2 cells. Treatment of C3H10T1/2 cultures with PD 98059, on the other hand, indicated a difference in the pattern of binding and activity of AP-1 which is dependent on cell density; unlike micromass cultures there is no correlation between the pattern of AP-1 binding and activity in monolayer cultures of C3H10T1/2 cells treated with increasing concentrations of PD 98059 inhibitor. Therefore, these findings indicate that first, the effect of ERK1/2 on AP-1 DNA binding in monolayer and micromass cultures are differently regulated, and secondly the DNA binding capability of AP-1 in monolayer cultures cannot be directly correlated to its activity. Whether the increase in AP-1 activity in monolayer cultures is due to an increase in phosphorylated active forms of the component of this transcription factor is the subject of further investigation.

Reverse transcription-PCR and promoter analyses indicate that an increase in cell–cell interaction, concomitant with an increase in c-Fos mRNA and reduced AP-1 and SRF activity, initiates a signaling program that leads to an increase in the chondrogenic gene expression. Our findings are also in agreement with and extend the studies done by Oh et al. which suggests a negative role for ERK in regulation of chondrogeneic genes as evidenced by induced expression of type IIB collagen and *Sox9* genes in PD 98059 treated micromass cultures. In addition, our findings indicate that as observed for chondrogenic cultures derived from early chick limb bud, expression of the aggrecan gene in chondrogenic cultures of C3H10T1/2 cells unlike the activity of type II collagen, Sox9 and c-Fos is a late event. Treatment of C3H10T1/2 micromass cultures with BMP-2 resulted in a great induction of aggrecan mRNA that is concomitant with an increase in collagen type IIB and *Sox9* gene activity, suggesting the involvement of BMP-2 in the advancement of the signaling program involved in chondrogenesis. Inhibition of BMP-2 treated or non-treated C3H10T1/2 cells with PD 98059 at different times in micromass cultures indicates that the activity of type IIB collagen, Sox9 and c-Fos are regulated differently and that the complexity of this regulation involves the time dependent cross interaction of MAP kinase and BMP-2 induced signaling activities. Interestingly, expression of type II A and B collagen genes is also detected in monolayer cultures indicating that C3H10T1/2 cells are more advanced in their chondrogenic fate than prechondrogenic cells of chick mesenchymal tissues. However, when C3H10T1/2 cells are cultured in micromass cultures, the ratio of type IIB to type IIA increases indicating a shift to more mature chondrogenic phenotype. It is also noteworthy that the activity of the aggrecan gene is reduced (more than fourfold) in 4.5-day micromass cultures which is concomitant with a 2.2-fold increase in the *c-Fos* gene expression. This finding is in line with the studies of Thomas et al. [2000] showing that overexpression of the *c-Fos* gene results in inhibition of chondrogenesis. In addition, ERK inhibition of 4.5 day micromass cultures treated with BMP-2 was observed to cause reduced levels of c-Fos (16% reduction) but enhanced aggrecan gene expression (threefold). Therefore, these findings in agreement with our previous results [Seghatoleslami and Tuan, 2002] and those of Thomas et al. [2000] suggest that increased levels of c-Fos beyond a certain threshold is inhibitory to chondrogenesis.

Overall, the data in this article indicate that changes in cell density lead to major alterations in the activity of ubiquitously acting signaling pathways such as that of MAP kinase as evidenced by a differential phosphorylation pattern of ERK1/2. These findings also indicate

that the effect of ERK1/2 on nuclear targets such as c-jun, c-Fos, and SRF is tightly controlled and possibly involves cross interaction with other signaling events that are activated by high cell density in micromass cultures. These changes in context of other signaling events such as that mediated by BMP-2 could in turn result in establishment of the molecular conditions necessary for cellular condensation, a prerequisite for the initiation and maintenance of chondrocyte specific gene regulation.

ACKNOWLEDGMENTS

The authors thank Genetics Institute for supplying recombinant human BMP-2. We thank Dr. Reza Bashey for critical reading of the manuscript.

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