

Identification of proteins differentially expressed during chondrogenesis of mesenchymal cells

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Abstract We performed comparative proteome analysis of mesenchymal cells and chondrocytes to identify proteins differentially expressed during chondrogenesis. Nine such proteins were identified. Type II collagen, matrilin-1, carbonic anhydrase-II (CA-II), 3'-phosphoadenosine 5'-phosphosulfate (PAPS) synthetase-2, and aldo-keto reductase were increased during chondrogenesis, whereas cellular retinoic acid binding protein-I (CRABP-I), CRABP-II, cytoplasmic type 5 actin, and fatty acid binding protein were decreased or almost disappeared. Expression of type II collagen, matrilin-1, PAPS synthetase-2, and CA-II was regulated by extracellular signal-regulated protein kinase, protein kinase C, and p38 kinase, signaling molecules known to regulate chondrogenesis.

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Key words: Chondrocyte; Chondrogenesis; Extracellular matrix; Mesenchymal cell; Signaling pathway

1. Introduction

Chondrogenesis is the process by which ectodermal mesenchymal cells differentiate into chondrocytes. Chondrogenesis is a key step in the development of cartilage and bone that occurs at limb buds during embryonic development. Initially, mesenchymal cells proliferate, increasing the number of chondrogenic competent cells. These cells subsequently undergo extensive cell-to-cell and cell-to-matrix interactions, leading to precartilage condensations, which are characterized by the denser packing of cells in particular regions. These precartilage condensations differentiate into cartilage nodules, in which the differentiated chondrocytes are located [1,2]. The process of chondrogenesis can be mimicked *in vitro* by culturing mesenchymal cells derived from limb buds at a high seeding density, such as in micromass culture.

Differentiated chondrocytes synthesize and secrete cartilage-

specific extracellular matrix (ECM) components, such as type II collagen and sulfated proteoglycan [3–5]. The balanced synthesis and degradation of cartilage-specific ECM molecules by chondrocytes are important for maintaining cartilage homeostasis, and the breakdown of this homeostasis results in degenerative diseases, including osteoarthritis and rheumatoid arthritis [6–8]. In addition, genetic defects in the chondrogenic pathway can cause many pathological symptoms, including dwarfism, chondrodysplasia, and osteoarthritis [9–11].

Chondrogenesis is regulated by complex protein kinase signaling pathways, which include protein kinase C α (PKC α) [12,13], extracellular signal-regulated protein kinase-1 (ERK-1) [14], and p38 mitogen-activated protein (MAP) kinase [15]. PKC α has been shown to positively regulate chondrogenesis of mesenchymal cells, in that the expression and activity of PKC α are dramatically increased during chondrogenesis, whereas the selective inhibition of this enzyme is sufficient to block this process [13,14,16]. The PKC α -dependent regulation of chondrogenesis appears to be exerted by inhibiting ERK-1 signaling [14]. In contrast, p38 kinase activity is increased during chondrogenesis in a PKC α -independent manner, and inhibition of its activity blocks chondrogenesis [15]. Therefore, the MAP kinase subtypes p38 kinase and ERK-1 oppositely regulate chondrogenesis by acting as positive and negative regulators, respectively, of this process.

To further elucidate the signaling pathways in chondrogenesis, we performed comparative proteome analysis to identify proteins differentially expressed by mesenchymal cells and chondrocytes. We identified nine proteins whose expression levels were changed more than two-fold. We proceeded to investigate the upstream signaling molecules for these nine proteins.

2. Materials and methods

2.1. Reagents

Antibody to type II collagen was purchased from Chemicon (Temecula, CA, USA). PD98059 was purchased from Biomol (Plymouth Meeting, PA, USA), Go6976 was purchased from Calbiochem (La Jolla, CA, USA), and SB203580 was obtained from Tocris (Ellisville, MO, USA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA), and Ham's F-12 medium was purchased from Gibco-BRL (Grand Island, NY, USA). The ECL detection system, GelBond PAGfilm, and Immobiline buffers were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Alcian blue 8GX, collagenase, α -cyano-4-hydrocinnamic acid, dithiothreitol (DTT), CHAPS, urea, iodoacetamide (IAA), and silver nitrate were purchased from Sigma (St. Louis, MO, USA).

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Abbreviations: AKR, aldo-keto reductase; CA-II, carbonic anhydrase-II; CRABP, cellular retinoic acid binding protein; ECM, extracellular matrix; FABP, fatty acid binding protein; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PMF, peptide mass fingerprinting

2.2. Micromass culture of mesenchymal cells

Mesenchymal cells derived from the distal tips of Hamburger–Hamilton (HH) 23/24 chicken embryo limb buds of fertilized white Leghorn chicken eggs were maintained as micromass cultures to induce chondrogenesis as described previously [14,15]. Briefly, cells, at a density of 2.0×10^7 /ml in Ham's F-12 medium containing 10% fetal bovine serum (FBS), were spotted as 15- μ l drops onto 35-mm or 60-mm culture dishes and incubated for 2 h at 37°C to allow attachment. Adherent cells were cultured in Ham's F-12 medium containing 10% FBS, 50 μ g/ml streptomycin, and 50 U/ml penicillin, and, where indicated, in the presence of PD98059 (10 μ M), Go6976 (1 μ M), or SB203580 (10 μ M). Chondrogenesis was monitored by Western blotting of type II collagen and by Alcian blue staining of sulfated proteoglycan [14,15].

2.3. Western blot analysis

Whole cell lysates were prepared by extracting proteins in sodium dodecyl sulfate (SDS)-sample buffer (62.5 mM Tris, pH 6.8, 5% mercaptoethanol, 10% glycerol, 2% SDS, 0.1% bromophenol blue). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) [17] and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 and incubated sequentially with antibody to type II collagen and peroxidase-conjugated secondary antibody, and the proteins were visualized with an ECL detection system.

2.4. Two-dimensional PAGE

Cells were washed three times with ice-cold phosphate-buffered saline, and total cell lysates were prepared by sonication in lysis buffer (9 M urea, 2% CHAPS, 0.8% pharmaryte, 1% DTT). The lysates were centrifuged at $15000 \times g$ for 30 min, and the supernatant was collected and stored at -80°C until use. Samples were applied to 18 cm long pH 4–9 IPG strips [18] for 12 h at 10 V using IPGphor. Isoelectric focusing was performed at 500 V for 1 h and at 1000 V for 1 h, and the voltage was gradually increased to 8000 V for 1 h. After focusing at 8000 V for another 4 h, each strip was incubated for 15 min in 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 1% DTT and for an additional 15 min in the same solution containing 2.5% IAA instead of DTT. The equilibrated strip was placed onto a 10–16% gradient polyacrylamide gel, and the gel was electrophoresed at 120 V in running buffer (25 mM Tris, pH 8.8, 192 mM glycine, 0.1% SDS).

2.5. Protein visualization and image analysis

The gels were fixed with 40% ethanol, 10% acetic acid for 1 h and with 5% ethanol, 5% acetic acid for 2 h. The gels were sensitized with 0.5 M sodium acetate, 1% glutaraldehyde for 30 min and stained with ammonial silver solution (47 mM silver nitrate, 0.33% ammonia, 20 mM sodium hydroxide) for 30 min. Proteins were developed with a solution of 0.01% citric acid, 0.1% formaldehyde, and the reaction was stopped with a solution of 5% Tris, 2% acetic acid. For in-gel digestion, the gels were fixed with 50% methanol, 12% acetic acid, 1.85% formaldehyde for 1 h, and with 50% ethanol for 2 h. The gels were pretreated with 0.02% sodium thiosulfate for 1 min, and impregnated with 0.2% silver nitrate, 2.8% formaldehyde for 30 min. After rinsing gels with deionized water, proteins were developed with a solution of 6% sodium carbonate, 1.85% formaldehyde, and the reaction was stopped with 50% methanol, 12% acetic acid.

All stained gels were scanned using a GS-710 densitometer (Bio-Rad, Hercules, CA, USA). The digitalized gel images were normalized and comparatively analyzed using PDQUEST program (v6.1).

2.6. In-gel digestion

Protein spots were excised from the gel and transferred into micro-centrifuge tubes. The gel pieces were washed twice with deionized/filtered water, 50 mM ammonium bicarbonate, and acetonitrile (ACN). Reduction and alkylation were carried out with 10 mM DTT and 55 mM IAA in 100 mM ammonium bicarbonate, respectively. The gels were again washed with 50 mM bicarbonate in 50% ACN for 30 min, dried thoroughly, and incubated for 16 h at 37°C in a solution of 50 ng/ μ l trypsin, 50 mM ammonium bicarbonate, 5 mM CaCl_2 . Each supernatant was transferred to a new tube, the gel was incubated with 25 mM ammonium bicarbonate in 50% ACN, and this supernatant was added to the same tube. The gels were then extracted twice with 5% formic acid in 50% ACN. All extracts of a gel piece were pooled and dried in a vacuum centrifuge.

2.7. Mass spectrometry and database search

The dried peptides were resuspended in 0.5% trifluoroacetic acid and mixed with matrix solution saturated with α -cyano-4-hydroxynamic acid. After drying the mixed sample on a plate, the mass of the peptide was determined using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Voyager DE-STR, Applied Biosystems). Internal calibration was performed with peptides from trypsin. Monoisotopic mass peaks were collected and applied to databases including Peptident (<http://expasy.nhri.org.tw/tools/peptident.html>) and MS-fit (<http://prospector.ucsf.edu/uscftml3.4/msfit.htm>) to identify the corresponding protein spots on 2D gels.

3. Results and discussions

3.1. Proteins differentially expressed in mesenchymal cells and chondrocytes

Chondrogenesis may be accompanied by a coordinated change of protein expression. To systematically determine the molecular mechanisms associated with chondrogenesis, we attempted to identify and characterize the proteins differentially expressed during this process. We therefore obtained mesenchymal cells from chick embryos of HH stage 23/24 by cutting the tip of the limb buds and spotting the cells onto plates. The cells differentiated into chondrocytes within 5 days as demonstrated by the accumulation of sulfated proteoglycan (Fig. 1A) and expression of type II collagen (Fig. 1B). When total proteins extracted from undifferentiated mesenchymal cells (day 1 cultures) and differentiated chondrocytes (day 5 cultures) were separated by 2D-PAGE, more than 2000 protein spots could be visualized by silver staining (Fig. 2A,B). We attempted to identify proteins differentially expressed more than two-fold in five independent experiments by peptide mass fingerprinting (PMF). Selected protein spots were excised from the gels, and the proteins were digested with trypsin. The peptides recovered from each protein spot were subjected to MALDI-MS. A database search, first of *Gallus gallus* proteins, and subsequently of proteins of other species, was used to identify the proteins. We identified nine proteins by PMF, with mass tolerances of 5–30 ppm and coverage of 5–76% (Fig. 2C, Table 1). Expression of five of these proteins – type II collagen, matrilin-1, carbonic anhydrase II (CA-II), aldo-keto reductase (AKR), and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) synthetase-2 – was increased or newly appeared during chondrogenesis. In contrast, expression of

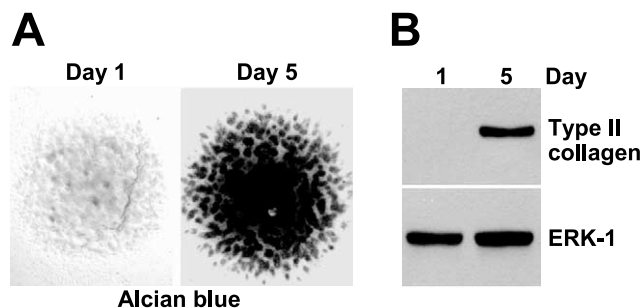


Fig. 1. Differentiation of mesenchymal cells into chondrocytes during in vitro micromass culture. Prechondrogenic mesenchymal cells were obtained from chick embryos of HH stage 23/24 by cutting the tip of the limb buds and spotting them on plates at a density of 2×10^7 cells/ml. A: Day 1 and day 5 cultured cells stained with Alcian blue. B: Total proteins from day 1 and day 5 cultured cells were separated by 7.5% 1D SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibody to type II collagen. ERK-1 was detected by immunoblotting as a loading control.

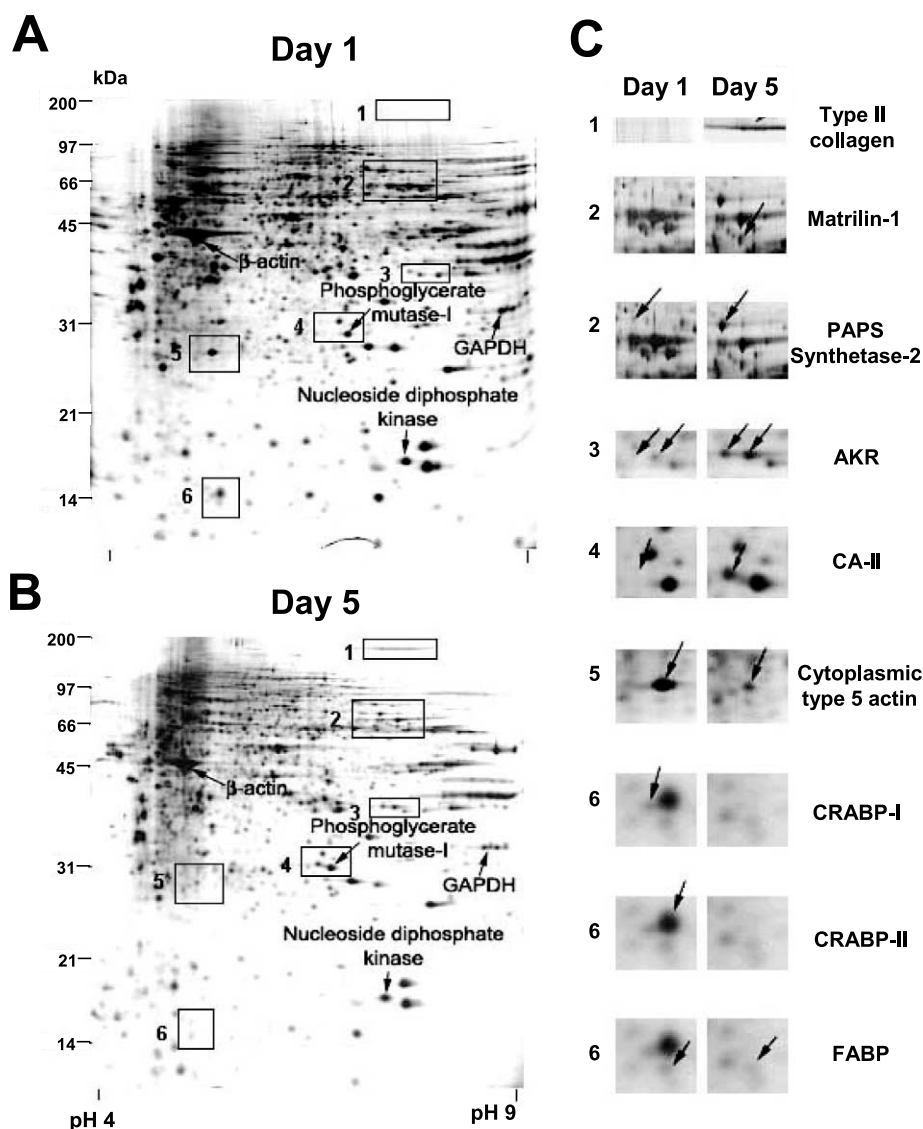


Fig. 2. Proteins differentially expressed during chondrogenesis. A,B: Total proteins from undifferentiated mesenchymal cells (day 1) and differentiated chondrocytes (day 5) were separated by 2D-PAGE using IPG strips of pH 4–9 and SDS 10–16% gradient gels. C: Comparative analysis of silver-stained gels. Ten protein spots in six different fields on 2D gels were selected as differentially expressed proteins and identified by PMF. Each 2D gel field is enlarged to more clearly show differential expression of each protein spot. The numbers indicate the corresponding regions in 2D gels.

four other proteins – fatty acid binding protein (FABP), cytoplasmic type 5 actin, cellular retinoic acid binding protein I (CRABP-I), and CRABP-II – decreased or almost disappeared in differentiated chondrocytes. Four protein spots were also identified by PMF as reference proteins: β -actin, phosphoglycerate mutase-I, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and nucleoside diphosphate kinase (Fig. 2A,B).

Of the five proteins increased or newly appearing in chondrocytes, four – type II collagen, matrilin-1, PAPS synthetase-2, and CA-II – were related to chondrocyte-specific ECM organization. Type II collagen, a biomarker of chondrocytes, was detected as a horizontal streak between pH 7 and pH 8, with an apparent molecular weight of 165 kDa, a pattern similar to that in bovine chondrocytes [19]. Matrilin-1, another cartilage matrix protein, is one of the most abundant non-collagenous fibrillar glycoproteins in cartilage matrix. This protein has been detected in several types of cartilage, includ-

ing articular cartilage and growth plate [20,21]. Although matrilin-1 knockout mice could survive, they had abnormalities in type II collagen fibrillogenesis and fibril organization in the matrix of the maturation zone [22]. CA-II is an enzyme that catalyzes the hydration and dehydration of carbon dioxide, making it important for pH balance and the calcification of cartilage [23]. PAPS synthetase-2 is an enzyme that produces the sole sulfur donor, PAPS [24]. PAPS synthetase null mice showed inhibited longitudinal growth, with a narrow range of the proliferating zone and reduced hypertrophy of chondrocytes [25]. Defects in the PAPS synthetase-2 gene result in many pathological symptoms, including human spondyloepimetaphyseal dysplasia and murine brachymorphism [9].

Three of the proteins whose expression is altered in chondrocytes – CRABP-I, CRABP-II, and AKR – are related to retinoic acid (RA) signaling or metabolism. RA is a potent regulator of growth plate chondrogenesis [26], and CRABPs are components of the RA signal pathway [27,28]. CRABP-II

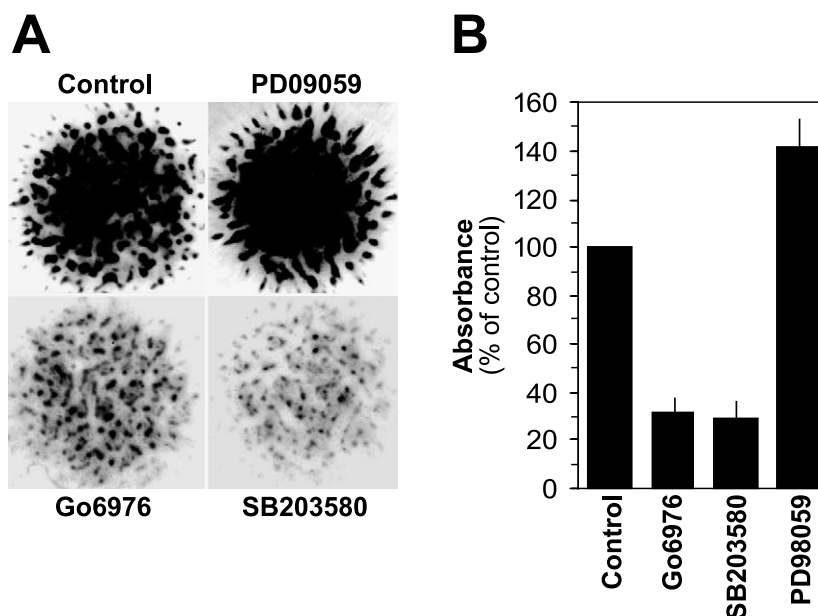


Fig. 3. Modulation of chondrogenesis by PD98059, Go6976, and SB203580. Mesenchymal cells were maintained as micromass culture for 5 days in the absence or presence of 10 μ M PD98059, 1 μ M Go6976, or 10 μ M SB203580. Accumulated sulfated proteoglycan was visualized by staining with Alcian blue (A) and quantified by measuring absorbance of the extracted dye at 600 nm (B). Data from three independent experiments were quantified and plotted as percentage of control.

directly channels RA to its receptor, facilitating RA signaling by translocating it into the nucleus [29,30]. CRABP-I, which is restricted to the cytosol, functions to sequester RA or to mediate its interaction with other metabolizing enzymes [31]. Since RA could induce both CRABPs [31], our finding that these proteins are down-regulated during chondrogenesis suggests that the concentration of endogenous RA decreases during this process. In addition, AKR, which catalyzes the reduction of retinaldehyde to retinal, can decrease the intracellular concentration of RA [32]. These findings indicate that RA plays a pivotal role in prechondrogenic mesenchymal cells, but that, during chondrogenesis, the cells gradually escape from RA signaling.

3.2. Regulatory signaling for the differential expression of proteins

Because chondrogenesis of mesenchymal cells is regulated by signaling cascades via various protein kinases, including ERK-1, PKC α , and p38 kinase, we examined the role of these

kinases in the differential expression of the identified proteins using specific inhibitors. Consistent with previous observations [14,15,33,34], chondrogenesis of mesenchymal cells was blocked by inhibition of PKC α with Go6976 or inhibition of p38 kinase with SB203580, whereas inhibition of ERK-1 by PD98059 enhanced chondrogenesis of mesenchymal cells (Fig. 3). To determine the role of each of these signaling molecules in the differential expression of proteins during chondrogenesis, cells were cultured in the presence of each specific kinase inhibitor, and the expression levels of the nine identified proteins were analyzed by 2D-PAGE (Fig. 4). Consistent with earlier reports [14,15], expression of type II collagen was increased in the presence of the ERK inhibitor PD98059, but decreased in the presence of the PKC α inhibitor Go6976 or the p38 kinase inhibitor SB203580 (Fig. 4). Expression of matrilin-1, PAPS synthetase-2, and CA-II was also increased by PD98059 and decreased by Go6976 or SB203580, indicating that these three proteins, as well as type II collagen, are downstream effector molecules of ERK-1, PKC α , and p38

Table 1
Proteins differentially expressed in mesenchymal cells and chondrocytes

Protein	Accession number	Observed		Mass		Expression level
		kDa	pI	Δ ppm ^a	% ^a	
Type II collagen	Q90W37	165	7.8	19.4	5	New
Matrilin-1	P05099	50	7.7	8.5	11	New
PAPS synthetase-2 ^b	O88428	66	7.5	30.1	10	Increase
AKR (1) ^c	Q90W83	36	7.5	11	29	New
AKR (2) ^c	Q90W83	36	7.7	5.4	24	Increase
CA-II	P07630	28	6.5	11.2	33	New
Actin, type 5	P53478	43	5.0	27.1	18	Decrease
CRABP-I	P40220	14.5	5.3	12.2	76	Decrease
CRABP-II ^b	P22935	15	5.6	21.6	42	Disappear
FABP	P80565	14	5.6	9.4	46	Decrease

^a Δ ppm, mass accuracy; %, coverage.

^bDatabase of mouse was used for PMF.

^cTwo different protein spots in 2D gel: acidic (1) and basic (2) variants.

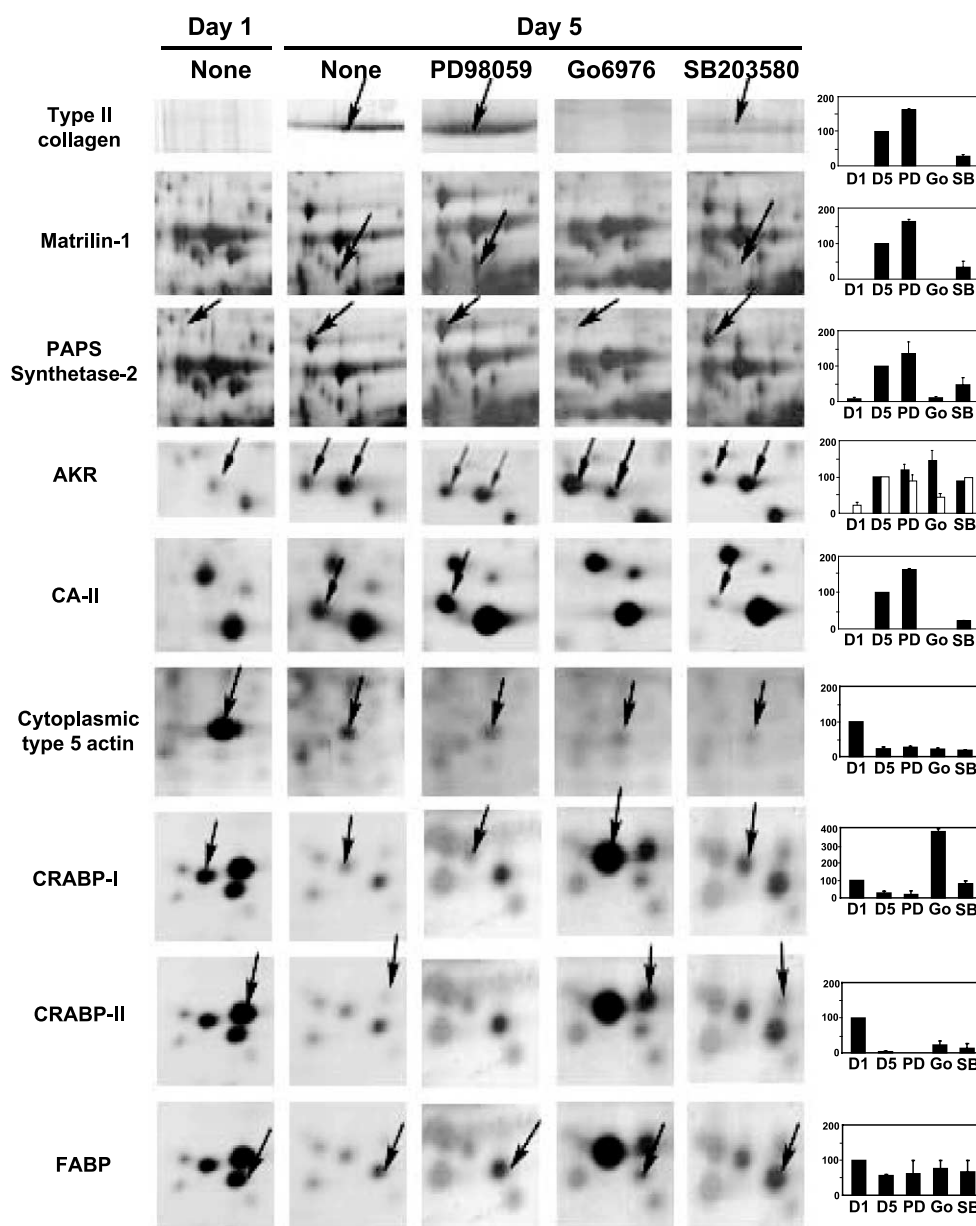


Fig. 4. Differential regulation of expression of the identified proteins by protein kinases. Cells were cultured for 5 days in the presence of 10 μ M PD98059, 1 μ M Go6976, or 10 μ M SB203580. Total protein extracts (80 μ g each) were separated by 2D-PAGE followed by silver staining. Spots representing the nine identified proteins from inhibitor-treated cells were compared with those from control cells through three independent experiments. The absence of an arrow from the box indicates an undetectable spot on silver-stained gels. Each protein spot was quantified using the PDQUEST program and data from three independent experiments were plotted as percentage of control (right panel). The intensity of protein spot in day 5 or day 1 culture is used as control (100%) for increased or decreased expression, respectively.

kinase. It is interesting to notice that the expression levels of four proteins – type II collagen, matrilin-1, PAPS synthetase-2, and CA-II – were uniformly increased and decreased by the treatment of PD98059 and SB203580, respectively. Since all four proteins are related to ECM organization of chondrocytes, these findings suggest a possibility that the genes of four proteins are regulated by the same chondrocyte-specific transcription factor(s) such as Sox5, 6 and 9 [35], which is under the control of these signaling cascades. Expression of AKR was not modulated significantly by treatment with PD98059 or SB203580. The two variants of AKR were differentially regulated by Go9676, in that expression of the acidic variant

was increased, whereas expression of the basic variant was decreased.

Protein kinase inhibitors modulated the expression of CRABP-I, CRABP-II, FABP, and cytoplasmic type 5 actin differentially. Although it was difficult to determine differences in expression level of these down-regulated proteins between control (day 5) and PD98059-treated cells, due to their low expression in control cells, we nonetheless found that expression of CRABP-II was reproducibly down-regulated by the ERK-1 inhibitor PD98059. The PKC α inhibitor Go9676 partially blocked down-regulation of CRABP-II, but it elevated the expression of CRABP-I more than three-fold compared

with day 1 mesenchymal cells. The p38 kinase inhibitor SB203580 significantly blocked down-regulation of CRABP-I and CRABP-II. It is interesting to notice that three proteins related to the RA signaling cascade – CRABP-I, CRABP-II and AKR – showed a partial response to PD98059, Go9676, and SB203580, which suggests that other signaling cascade(s) may be involved in the regulation of these RA-related proteins. In contrast, inhibition of the three protein kinases resulted in no noticeable changes in the expression of cytoplasmic type 5 actin and FABP. Since there was the discrepancy between these protein levels and chondrogenic phenotype (Figs. 2 and 4), and the roles of these proteins in chondrogenesis are not clear, the observed decrease of cytoplasmic type 5 actin and FABP may be secondary effects for chondrogenesis.

Taken together, these results demonstrate that PKC α , ERK-1, and p38 kinase are major upstream regulators of chondrogenesis and of proteins differentially expressed during this process. Our results also showed that expression of some differentially expressed proteins were not affected by the inhibition of PKC α , ERK-1, or p38 kinase, suggesting the existence of other signaling or regulatory molecules for the differential expression of these proteins.

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