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Effect of cartilage oligomeric matrix protein on mesenchymal chondrogenesis *in vitro*

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Summary

Objective: Cartilage oligomeric matrix protein (COMP) mutations have been identified as responsible for two arthritic disorders, multiple epiphyseal dysplasia (MED) and pseudoachondroplasia (PSACH). However, the function of COMP in chondrogenic differentiation is largely unknown. Our investigation focuses on analyzing the function of normal COMP protein in cartilage biology.

Methods and results: To explore the function of COMP we make use of an *in vitro* model system for chondrogenesis, consisting of murine C3H10T1/2 mesenchymal cells maintained as a high-density micromass culture and stimulated with bone morphogenetic protein 2 (BMP-2). Under these culture conditions, C3H10T1/2 cells undergo active chondrogenesis in a manner analogous to that of embryonic limb mesenchymal cells, and have been shown to serve as a valid model system to investigate the mechanisms regulating mesenchymal chondrogenesis. Our results indicate that ectopic COMP expression enhances several early aspects of chondrogenesis induced by BMP-2 in this system, indicating that COMP functions in part to positively regulate chondrogenesis. Additionally, COMP has inhibitory effects on proliferation of cells in monolayer. However, at later times in micromass culture, ectopic COMP expression in the presence of BMP-2 causes an increase in apoptosis, with an accompanying reduction in cell numbers in the micromass culture. However, the remaining cells retain their chondrogenic phenotype.

Conclusions: These data suggest that COMP and BMP-2 signaling converge to regulate the fate of these cells *in vitro* by affecting both early and late stages of chondrogenesis.

Key words: Cartilage oligomeric matrix protein, Cartilage, Arthritis, Chondrogenesis.

Introduction

Cartilage oligomeric matrix protein (COMP) is a 524 kDa oligomeric glycoprotein[1,2]. It is a member of the thrombospondin gene family[2,3] and has recently been termed thrombospondin 5. The mature protein is a pentamer of identical subunits that interact at their N-terminus through disulfide linkages, forming a bouquet-like structure[1,4,5]. The pentamer is formed from an alpha-helical coiled coil structure located at the amino terminus of each monomer[3,4,6]. COMP contains both epidermal growth factor-like repeats and calcium-binding repeats located in the central region of the protein[3]. These repeats are contained in all the thrombospondins[3].

COMP was originally identified as a component of cartilage and has been found to be highly expressed in both developing and mature cartilage as well as in tendon and ligament[1,7–9]. During fetal development, COMP expression was pronounced in the territorial matrix around the chondrocytes of the growth plate[8]. However, in adult cartilage, COMP has been shown to be located primarily in the interterritorial matrix[8]. Since COMP is localized to the territorial matrix of the chondrocytes, it has been proposed that it may directly interact with cells, which has been recently shown to be the case[10]. But whether COMP plays a direct role in the control of chondrocyte proliferation and differentiation is not yet known.

While the function of COMP in cartilage is not yet known, mutations in the COMP gene have been identified that are responsible for at least two forms of heritable osteoarthritis (OA), multiple epiphyseal dysplasia (MED or EDM1) and pseudoachondroplasia (PSACH)[11,12]. Although it is not currently known if the onset of OA is secondary to poor joint formation or if it is a primary result of the action of the mutated COMP protein, it is known that many of the mutations in COMP reside in the calcium-binding region[11,12]. It now appears that these mutant COMP molecules are indeed defective in binding calcium[10]. Since the initial identification of COMP as the causative agent in MED and PSACH, a large number of mutations have been identified in the COMP gene in these patients[13–19]. These findings have led to the postulate that certain features of heritable as well as sporadic OA may have roots in the noncollagenous components of cartilage such as COMP. This, however, remains to be determined.

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While it is likely that COMP plays an important role in cartilage biology, the nature of this role is unknown. To better understand the normal function of COMP in cartilage biology, we have utilized an *in vitro* mesenchymal chondrogenesis model system, consisting of the pluripotential murine mesenchymal cell line (i.e. C3H10T1/2 cells). These cells are able to undergo chondrogenesis in micromass culture following treatment with bone morphogenetic protein-2 (BMP-2)[20–23]. We report here that ectopic expression of COMP in these cells by retroviral gene transfer induces a number of effects on BMP-2-mediated chondrogenesis in this system.

Methods

CELL CULTURE, TRANSFECTIONS, INFECTIONS AND BMP-2 TREATMENT

C3H10T1/2 mesenchymal fibroblasts and ecotropic Phoenix packaging cells were obtained from ATCC and monolayer cultures were maintained in DMEM supplemented with 10% fetal bovine serum (FCS, Atlanta Biologicals), 50 U/ml penicillin and 50 mg/ml streptomycin. The cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2. The medium was changed every 3 days. All transfection experiments were initiated on 50% confluent monolayer cultures. Plasmids (30 µg) were transfected by the calcium phosphate procedure. The human COMP cDNA was cloned into the Eco R1 site of the pBABE retroviral vector. When transfected into Phoenix cells this plasmid is converted to infectious retrovirus. Following the transfection of the pBABE constructs, the Phoenix cells were placed in a 32°C humidified incubator for 48 h, since the virus is more stable at 32°C. The medium containing infectious virus was harvested and filtered through a 0.2 µm filter to eliminate any contaminating cells. The virus was then used to infect subconfluent, proliferating C3H10T1/2 fibroblasts in the presence of 4 µg/ml of Polybrene (Sigma).

The micromass culture technique was modified from the work of Ahrens *et al*.[24] as in Denker *et al*.[25]. Cells were trypsinized and resuspended in Hams F12 medium with 10% FCS at a concentration of 10^7 cells/ml. A 10 µl drop of this cell suspension was placed in either a 10 cm polystyrene tissue culture plate (Corning) or the center of a well of a 24-well polystyrene tissue culture dish (Corning). The cells were allowed to adhere for 2 h at 37°C and then medium containing 10% FCS, with or without added BMP-2 (100 ng/ml, a generous gift from the Genetics Institute, Inc., Cambridge, MA, USA), as indicated subsequently. The media were changed every 3 days.

HISTOLOGY, METABOLIC SULFATE LABELING AND FLOW CYTOMETRY

To measure the production of sulfated glycosaminoglycans, compared with total protein, sodium $^{35}SO_4$ and ^{3}H-leucine (NEN, Boston, MA), were added to the medium at final specific activities of 5 and 1 µCi/ml, respectively, at the times indicated below. After incubation for an additional 24 h, the medium was removed and cells were rinsed extensively with PBS, and fixed in 4% paraformaldehyde. Radioactive incorporation of $^{35}SO_4$ and ^{3}H-leucine was assessed by liquid scintillation counting.

Whole-mount cultures were rinsed twice with phosphate buffered saline (PBS), fixed for 20 min in 4% paraformaldehyde and then as indicated below, stained with a 1% solution of Alcian Blue 8-GX (Sigma, St. Louis, MO) at pH 1. Sections were immunostained for collagen type II using monoclonal antibody CIIC1 as described previously[20]. Following deparaffinization and rehydration, sections were digested with 0.5 mg/ml bovine testicular hyaluronidase in 10 mM NaH_2PO_4/KH_2PO_4, at pH 6 in the presence of 6-aminocaproic acid, 1 mM phenylmethylsulfonlyl fluoride (PMSF) and 5 mM benazamidine hydrochloride overnight, then swelled in 0.5 M acetic acid for 5 h. Incubations with primary antibody (42 µg/ml in 10% goat serum in PBS) for 60 min at 37°C were followed by rinsing with PBS and incubation for 20 min with biotin-conjugated broad-spectrum anti-IgG secondary antibody. Visualization was performed using streptavidin-conjugated horseradish peroxidase Histostain-SP Kit (Zymed Laboratories) and ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL).

For cell cycle analysis (flow cytometry), cells were rinsed once in chilled PBS then trypsinized and resuspended in 10 ml DMEM plus 10% FCS. The cells were pelleted and resuspended in 70% ethanol. The cells were kept on ice for 10 min, pelleted and then treated with RNAase A (180 µg, Sigma) for 30 min at room temperature. Propidium iodide (Sigma) was added to a final concentration of 75 µg/ml. Cell cycle analysis was performed on a Coulter Profile 2, Flow Cytometer.

RNA ISOLATION, NORTHERN AND RT-PCR ANALYSIS

Total RNA was isolated from cells by the Trizol method (Gibco-BRL). Oligo dT was used as the primer in the reverse transcription reaction which was followed by a PCR reaction with the appropriate primers. For the RT-PCR reactions, 1 µg of total RNA from the cells was used in each reaction. All total RNA samples were DNase I treated in the RT reaction prior to the PCR reactions. Additionally, as a control, PCR done in the absence of RT was negative for any ethidium bromide stained bands (data not shown).

PROTEIN ANALYSIS AND IMMUNOBLOTTING

Cells were cultured in serum-free medium for 6–8 h. The medium was harvested and the cellular debris was removed by centrifugation. Aliquots of the medium were added to two volumes of ice-cold acetone and the proteins were precipitated at –20°C, as in Jordan-Sciutto *et al*.[26]. The protein precipitate was pelleted by centrifugation at 14,000×*g* for 10 min at 4°C. The protein precipitate was then boiled in SDS-PAGE sample buffer in the presence of reducing agent (DTT). For immunoblotting, the proteins were electrophoretically resolved by SDS-PAGE (40 µg protein/lane) and transferred onto nitrocellulose filters. The blots were then washed in TBST buffer (10 mM Tris, pH 8; 150 mM NaCl; 0.05% Tween 20), blocked with 2.5% bovine serum albumin (BSA) in TBST for 30 min at room temperature, and incubated with the primary antibodies for 60 min at room temperature in TBST. Anti-human COMP antibody (a kind gift of Dr Heinegard) was used at a concentration of 1:500. The blot was then washed three times, 10 min each, in TBST, then incubated with a 1:7500 dilution of secondary antibody conjugated to alkaline phosphatase, for 30 min at room temperature in TBST. The blot was then processed using the Protoblot system (Promega).

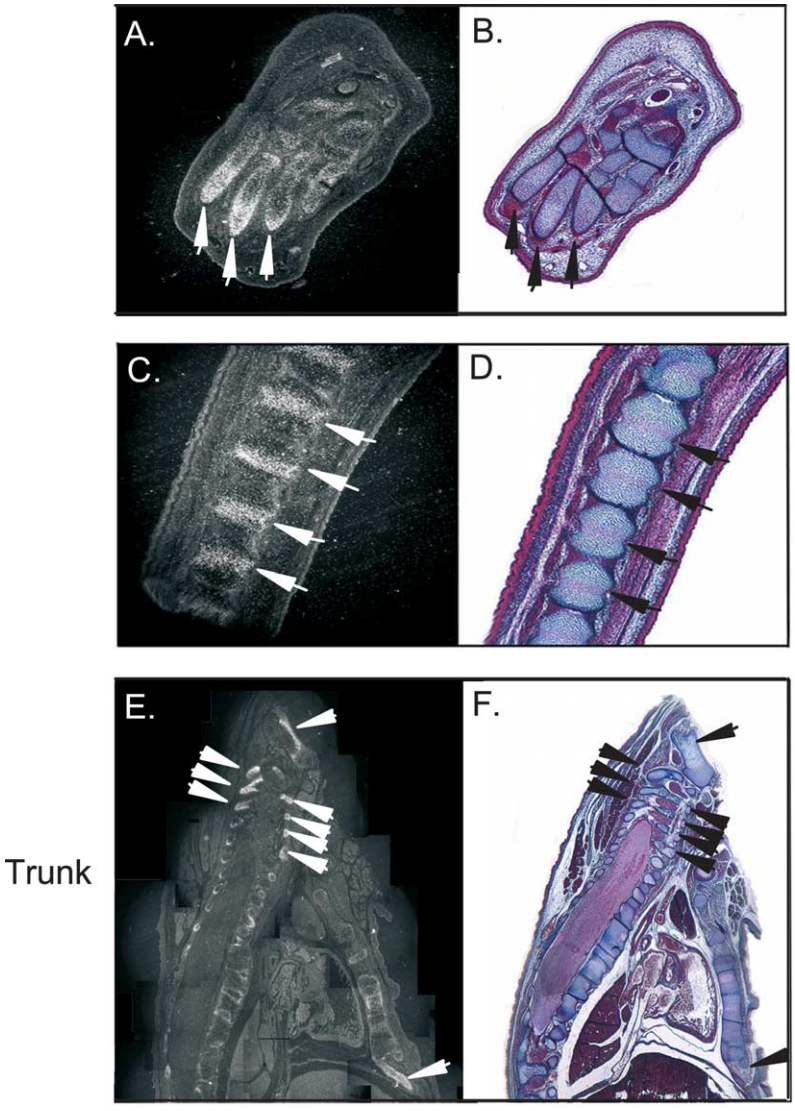


Fig. 1. *In situ* hybridization demonstrates widespread COMP expression in the developing cartilage of Day 16.5 embryonic mouse. Sagittal sections of embryonic mice (day 16.5 post coitus dpc) were processed for *in situ* hybridization for COMP mRNA, as described in the Methods section. The sections were observed by darkfield optics to visualize the *in situ* hybridization signal (left column), or by bright field optics to view the histology by Mallory's Trichrome stain (right column). Three regions of the embryo are shown: (A,B) footpad, (C,D) tail vertebrae and (E,F) trunk. Positive hybridization signals (white grains indicated by the arrows) were localized to sites of active chondrogenesis in the developing skeletal elements of the digits of the paw (A), the tail (C) and the vertebral column and sternum (E). The data show that COMP expression is extensive in the regions undergoing chondrogenesis. The black arrows (B, D and F) indicate the corresponding regions in the control, stained sections. Controls using radiolabeled sense probe showed no detectable hybridization signal (data not shown).

IN SITU HYBRIDIZATION

Embryonic mice (16.5 dpc) were fixed, embedded and sagittally sectioned, as in Wawersik and Epstein[27]. The sections (8 μM) on glass slides were then processed for *in situ* hybridization using a ^{35}S-labeled mouse COMP probe. The probe (either antisense or sense riboprobe) was generated with a fragment of the mouse COMP cDNA spanning nucleotide residues 1409–2051 (642 bp). The sections were hybridized (1×10^6 cpm total) to probe (55°C), washed, RNase treated, immersed in photographic emulsion and then processed for autoradiography at Day 10. The autoradiographs of the probed sections were photographed under darkfield optics where a positive hybridization signal is evident as white grains. Additionally, near adjacent sections were stained for histology using Mallory's Trichrome. As controls, the radiolabeled sense probe showed no detectable hybridization (data not shown).

Results

EXPRESSION OF COMP DURING MOUSE EMBRYOGENESIS IN DEVELOPING CARTILAGE

Using the cloned murine COMP cDNA[28], we localized COMP expression in the mouse embryo undergoing active chondrogenesis and cartilage development at Day 16.5 pc. As shown in Fig. 1, a high-level expression of COMP was seen at multiple sites in the footpad, trunk and tail and in particular, regions very active in cartilage formation. These included the developing digits in the

footpad, the tail vertebrae, and spine, ribs and sternum all corresponding to sites involved in endochondral ossification. This tissue distribution clearly indicates that COMP expression is part of the cartilage development program.

INDUCTION OF CHONDROGENESIS *IN VITRO* BY BMP-2 USING C3H10T1/2 CELLS

Since COMP is expressed to high levels during embryonic development, it has therefore been assumed that COMP plays some role in cartilage development. However to date, this role is not known. To examine the role of COMP during chondrogenesis, an *in vitro* culture system, of C3H10T1/2 mouse mesenchymal cells was therefore used in the studies described here. These multipotential cells are able to differentiate in culture along multiple pathways depending on the stimulus[29]. For example, they are capable of differentiating into myoblasts, osteoblasts, adipocytes or chondrocytes under the appropriate culture conditions[20–25,29–32]. Importantly, it has been shown that these C3H10T1/2 cells can preferentially undergo chondrogenesis when cultured in a high-density micromass environment in the presence of BMP-2[20,21,25]. This culture system presents a useful model to study COMP function, since the effect of misexpression of COMP on chondrogenesis can therefore be analyzed *in vitro*.

An example of the chondrogenic differentiation of these C3H10T1/2 cells is shown in Fig. 2A, where the cells spotted in a micromass (10^7 cells/ml in a 10 µl drop) and treated with BMP-2 show prominent positive staining with Alcian Blue by Days 7–10 in culture. This positive staining indicates the presence of highly negatively charged sulfated proteoglycans of cartilage matrix[33], which is accompanied by a corresponding increase in metabolic $^{35}SO_4$ incorporation into sulfated proteoglycans [Fig. 2(B)]. RT-PCR analysis shows that there is a clear increase in the level of type II collagen (both the 'A' and the 'B' splicing isoforms) [Fig. 2(C)]. The increase in the IIB isoform indicates the presence of differentiated cartilage[34,35]. These chondrogenic markers are not seen in monolayer cultures of these cells treated with BMP-2 (references 20,21,25 and data not shown). When the micromass cultures were sectioned and examined by immunohistochemistry, collagen type II was detected in the sections, as shown in Fig. 2D. The intensity of the staining in the BMP-2 stimulated cultures was considerably higher than the control cultures, indicating an abundance of matrix [Fig. 2(D)]. In this regard, we have also previously shown significant increases in the level of cartilage proteoglycan link protein in the BMP-2-treated micromass cultures, compared with the untreated cultures[20].

RETROVIRAL GENE TRANSFER OF COMP IN CULTURES OF C3H10T1/2 CELLS

To determine the effect of COMP on chondrogenesis, we ectopically expressed COMP in C3H10T1/2 cells and then

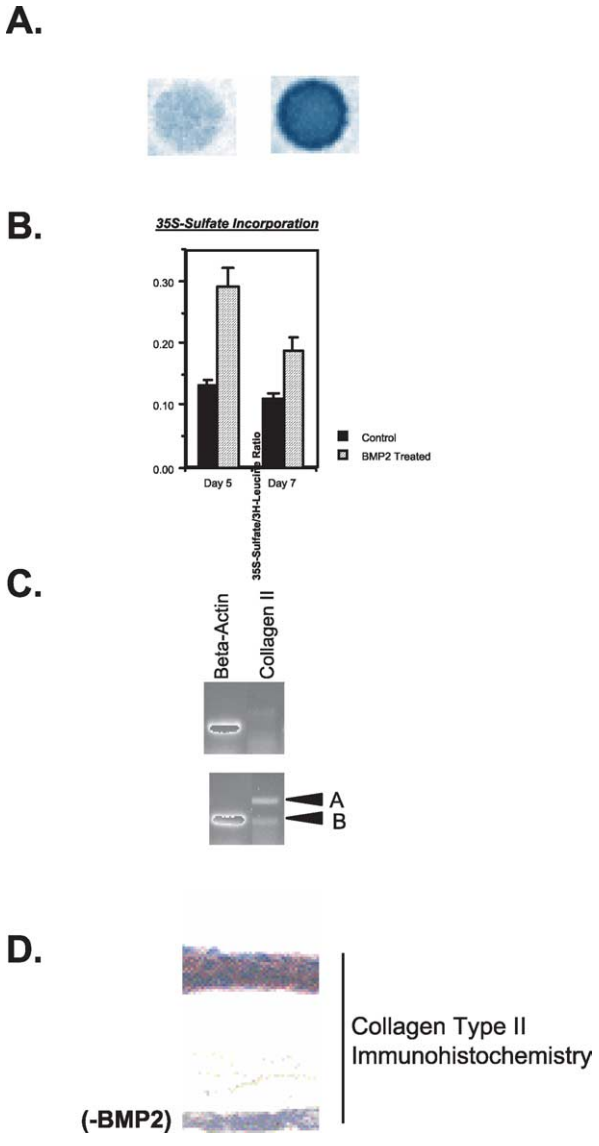


Fig. 2. BMP-2 treatment of micromass cultures of C3H10T1/2 cells leads to induction of chondrogenesis. C3H10T1/2 cells were cultured in a micromass environment in the presence or absence of 100 ng/ml of BMP-2, as described in the Methods section. (A) Alcian Blue stain. Alcian Blue staining of paraformaldehyde fixed, Day 5 cultures reveals a significant level of chondrogenesis in the BMP-2-treated micromasses. (B) Metabolic sulfate incorporation. $^{35}SO_4$ was added to the micromass cultures (5 µCi/ml final concentration) for a 24 h period ending at Day 5 or 7. ^{3}H-leucine at 1 µCi/ml (final concentration) was also added as a control to standardize for differences in protein levels. At the end of the labeling period, the cultures were washed extensively in PBS to remove any unincorporated label and then fixed in 4% paraformaldehyde. The amount of radioactive incorporation in each micromass was determined by scintillation counting. The ratio of $^{35}SO_4$ to ^{3}H-leucine incorporation on Days 5 and 7 is shown. The data show higher sulfate incorporation in the BMP-2-treated cultures (slashed bars) compared with controls (solid). (C) RT-PCR analysis. Total RNA isolated on Day 5 of culture was processed for RT-PCR using primers specific for mouse collagen type II and mouse β-actin. The primers for type II collagen differentiate between the 'A' and 'B' forms of the transcript (the 'B' form is cartilage specific). The reaction products, as shown, were electrophoresed on an agarose gel, which was stained with ethidium bromide. The stained gel shows a higher level of expression of both collagen type II transcripts in the BMP-2-treated cultures relative to the levels of β-actin mRNA. (D) Immunohistochemistry. Day 7 micromass cultures were processed for immunohistochemistry of collagen type II and revealed a significant level of this cartilage matrix protein in the BMP-2-treated cultures, compared with untreated controls. Collagen type II is detected by HRP staining (shown as red), and the cells were counterstained with hematoxylin (blue).

assessed its effect on chondrogenesis, following micromass culture of these cells. We first determined the level of endogenous COMP expression in these cells, prior to any ectopic expression of COMP. C3H10T1/2 cells were found to express no detectable COMP, either by Northern blot analysis or by RT-PCR (using primers specific for the mouse COMP sequence; reference 28). These cells therefore represent an excellent cell line to test the effects of ectopic COMP expression since they are effectively null for COMP expression. The human COMP cDNA was then cloned into the pBABE retroviral vector, which produces a replication defective virus, which was then transfected into the Phoenix ecotropic packaging cell line. As a control, the retroviral plasmid alone was separately transfected into the Phoenix cells. The medium containing the infectious virus was harvested, filtered and used to infect subconfluent (20%), proliferating C3H10T1/2 mesenchymal fibroblasts, in the presence of Polybrene to enhance the infection. The cells were then grown to confluence for an additional 72 h. Under these conditions, the infection level is approximately 90–95%, based on X-gal staining of β-galactosidase activity produced by expression of *LacZ* in the pBABE retroviral vector control (data not shown).

Following outgrowth of the infected cells, they were first monitored for expression of COMP. As shown in the RT-PCR analysis in Fig. 3A, COMP is expressed at a high level in the cells infected with pBABE-COMP retrovirus as opposed to the control-infected culture. Immunoblot analysis of secreted proteins from the cells grown in serum-free medium showed expression of COMP protein [Fig. 3(B)]. As shown in Fig. 3B, the asterisk denotes a protein detected by the secondary antibody, whose levels appear to be elevated in the COMP expressing cells. One possibility for the elevated levels is that COMP may positively affect secretion of other proteins from the cell. In this regard we do see some elevation in other high-molecular proteins in COMP-infected cells (Hall, unpublished observations).

Taken together, these data indicate that the retroviral transfer of COMP results in a detectable level of ectopic expression. Since mutant COMP expression is known to lead to an altered cellular morphology, and in particular an enlarged and distended endoplasmic reticulum[36–38], it was important to determine if this altered morphological phenotype was present in the COMP expressing cells. As seen in Fig. 3C, the COMP expressing cells are morphologically

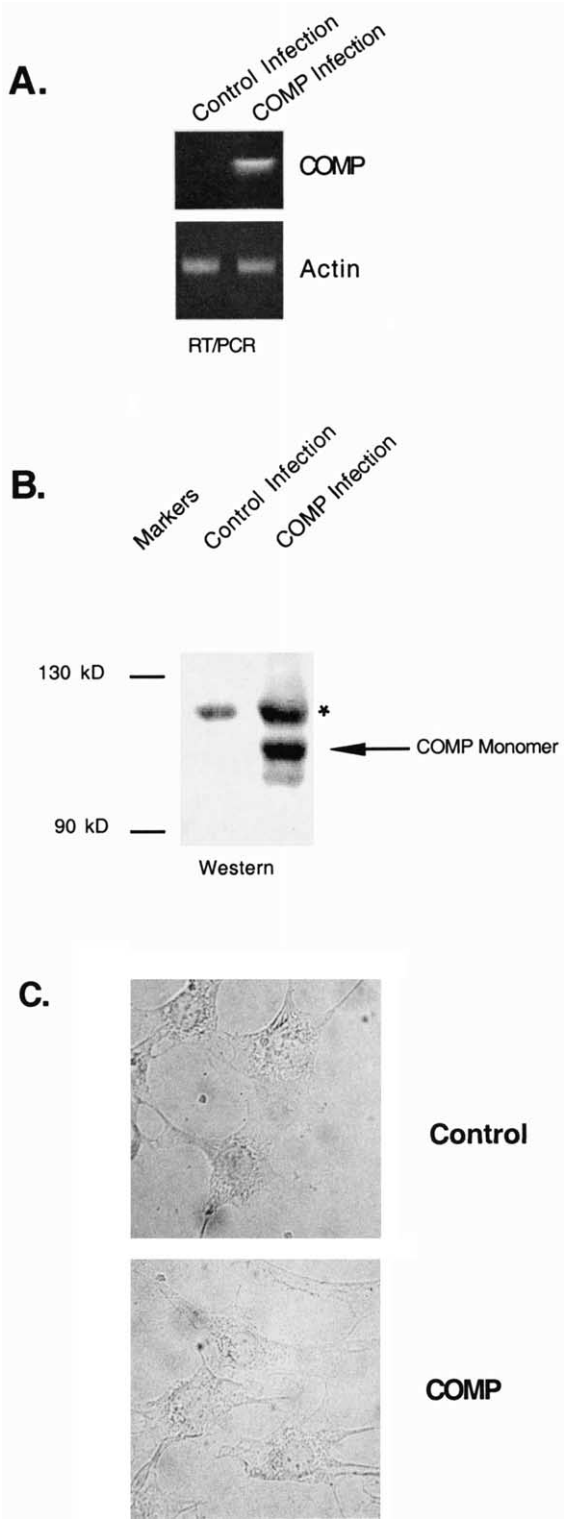


Fig. 3. Ectopic expression of COMP by retroviral gene transfer in cultures of C3H10T1/2 mesenchymal cells. (A) RNA analysis. RT-PCR was performed on 1 μg of total RNA isolated from confluent monolayer cultures of murine C3H10T1/2 cells at 7 days postinfection with either the control (pBABE) or the COMP expressing (pBABE-COMP) retrovirus. Also included was RNA from uninfected control C3H10T1/2 cells. The RT-PCR reaction was performed with primers specific for either COMP or β-actin and the reaction products were electrophoresed on an agarose gel, stained with ethidium bromide. The bands corresponding to COMP and β-actin are indicated and show a significant level of ectopic COMP expression in the pBABE-COMP infected culture but no detectable expression in the control. (B) Immunoblot analysis. The cells at 7 days postinfection with either the control or COMP retrovirus, as in (A), were cultured in serum-free media for 12 h. The media were harvested, the protein precipitated in cold acetone, which were analyzed by SDS-PAGE in the presence of reducing agent (DTT). The SDS-PAGE gel was immunoblotted and the blot incubated with an antibody generated against human COMP (the secondary antibody was conjugated to alkaline phosphatase). (Left side) The band corresponding to the COMP monomer protein is evident only in the extracts of the pBABE-COMP infected cells. (Right side) An identical immunoblot was incubated with secondary antibody only. The asterisk denotes a protein detected by the secondary antibody. (C) Phase contrast microscopy. Monolayer cells infected with control and COMP retrovirus (7 days postinfection), as in (A), were photographed by phase contrast microscopy. The cells infected with each retrovirus are indicated. The results revealed no significant differences in the phenotype between the control and COMP expressing cells.

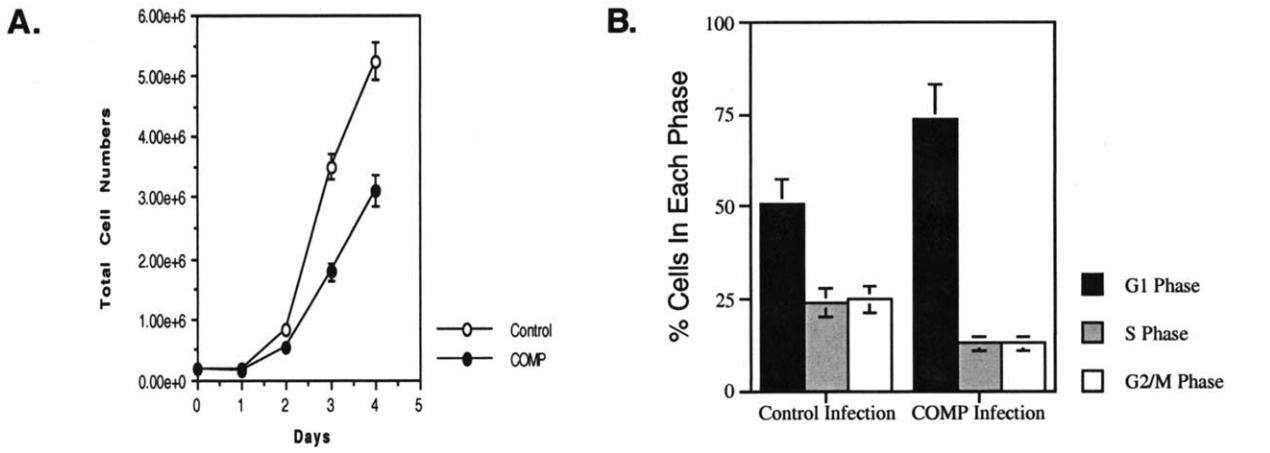


Fig. 4. Cells expressing COMP display a slow growth phenotype with increased numbers of cells in the G1 phase of the cell cycle. C3H10T1/2 cells infected with control and COMP expressing retrovirus, as in Fig. 3, were seeded at 100,000 cells/10 cm plate in monolayer. At the indicated days, the cells were trypsinized and processed for viable cell counts (by trypan blue exclusion) and flow cytometry was performed. Shown in (A) are the total viable cell numbers/plate on the indicated days. Shown in (B) are the cell cycle distributions taken at Day 3 of cell culture. The data show that compared with the control (open circle), COMP expression (solid circle) leads to a slower growth rate with a correspondingly increased number of cells in G1 phase and fewer cells in S/G2/M phases.

identical to the control cells. This indicates that COMP expression does not adversely affect cell structure or morphology (i.e. no obvious increase in cell size or number of subcellular vesicles are evident in the COMP expressing cells).

ECTOPIC COMP EXPRESSION AFFECTS CELL GROWTH AND CHONDROGENESIS *IN VITRO*

To determine the functional consequences of this ectopic expression, the cells infected with either the control virus or the COMP expressing virus were cultured in a monolayer. Since COMP protein is present in the territorial zone of proliferative chondrocytes in the growth plate during development[7], it is possible that COMP functions in part to regulate the extent of chondrocyte proliferation. To assess the growth regulatory function of COMP in culture, the cultures were seeded at equal cell density and processed for viable cell counts and flow cytometry at daily intervals thereafter. As seen in Fig. 4A, the cells expressing COMP had a demonstrable slow growth phenotype, evident as a twofold decrease in cell number by Day 4 of culture. This reduction in the level of cell proliferation was mirrored by a twofold decrease in the percent cells in both S and G2/M phases with a corresponding increase in the percent cells in G1 phase [Fig. 4(B)]. Thus, COMP appears to reduce the extent of cell proliferation in monolayer cultures by changing the cell cycle distribution pattern, resulting in more cells in G1 phase.

Next, the cells infected with either the control virus or the COMP expressing virus were cultured in a high-density micromass environment and then treated with or without BMP-2 (100 ng/ml). The cultures were fixed at Day 10 and stained with Alcian Blue. As shown in Fig. 5A, BMP-2-treated cells expressing COMP were stimulated to undergo chondrogenesis to a higher level than the control cells treated with BMP-2, as evident by increased Alcian Blue staining. When the intensity of the stain was quantified by scanning densitometry, it was found that the COMP expressing cells showed nearly a twofold increase in the level of Alcian Blue staining over the control cells [Fig. 5(B)]. That COMP expression enhanced BMP-2-mediated chondrogenesis was also evident by measuring metabolic $^{35}SO_4$ incorporation into extracellular matrix. As seen in Fig. 5C, by Day 10 of culture the relative level of $^{35}SO_4$ incorporation in the COMP-infected cells was higher than that in the control cells following BMP-2 treatment. This increase in $^{35}SO_4$ incorporation appears to be a time dependent event, since the levels of $^{35}SO_4$ incorporation at early time points (i.e. Days 4 and 7) were nearly equal between the control and COMP expressing cells.

To assess specific markers of chondrogenesis, the transcript levels of aggrecan and type II collagen were measured by RT-PCR. As seen in Fig. 5D, the levels of both genes were increased by BMP-2 at Day 6. COMP had only a minor effect on enhancing the expression of these genes above that of BMP-2. Fig. 5E shows a control at Day 10, where expression of both the A and B forms of collagen type II are increased in the BMP-2 stimulated micromass culture. In total, these observations indicate that COMP may only enhance certain aspects of chondrogenesis, such as those reflected by Alcian Blue staining and sulfate incorporation.

IN THE PRESENCE OF BMP-2, COMP EXPRESSION CAUSES AN INCREASE IN APOPTOSIS AND A LOSS OF CELLS IN THE MICROMASS CULTURE

Since the data in Fig. 5 indicated that COMP enhanced some aspects of chondrogenesis (i.e. by sulfate incorporation and Alcian Blue staining) but only at Day 10 of culture, later cultures were therefore examined to assess the long-term effects of COMP. Control and COMP expressing micromasses, with and without BMP-2 treatment as described in Figs. 2 and 5, were examined to Day 16. Interestingly, we observed that on Days 13 and 16, the COMP expressing micromass cultures treated with BMP-2 have lost all Alcian Blue staining, while the control culture treated with BMP-2 retained its staining intensity (shown in Fig. 6). This indicates that either the cultures had lost a majority of their sulfated proteoglycan-rich matrix or that the cultures had a dramatic reduction in the numbers of cells.

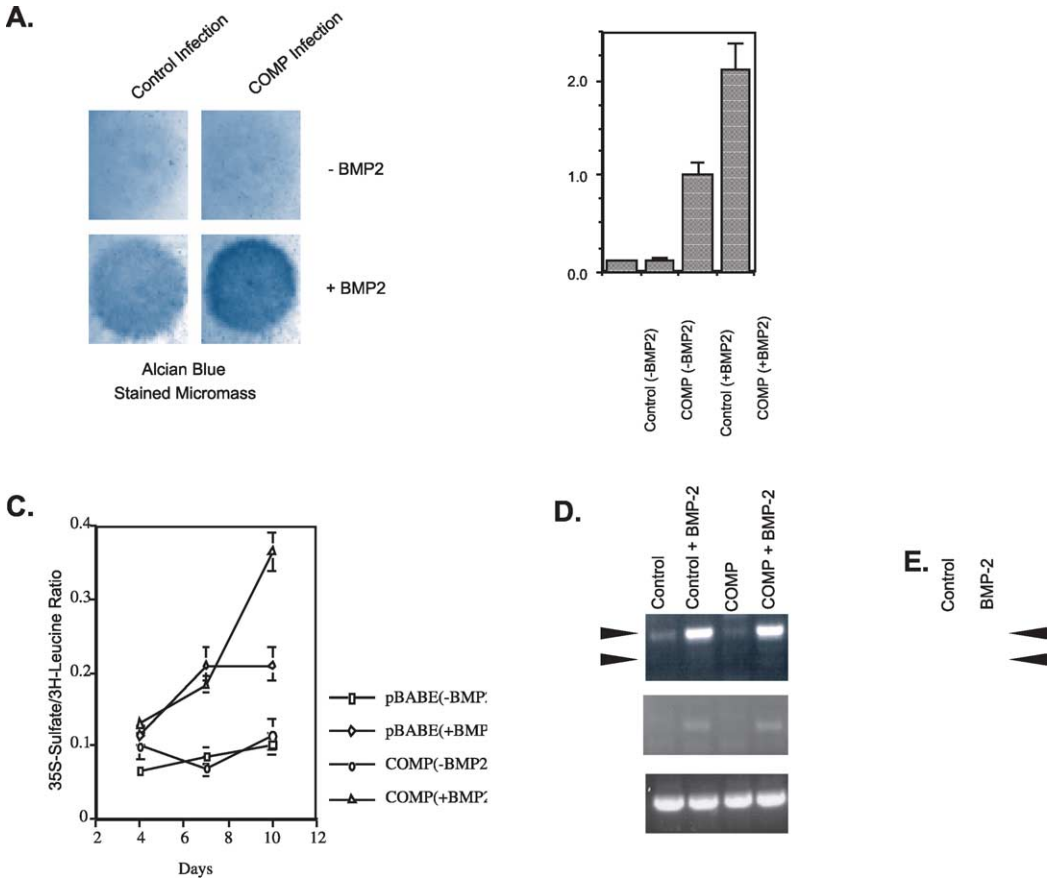


Fig. 5. Retroviral expression of COMP in cultures of BMP-2-treated C3H10T1/2 mesenchymal cells stimulates chondrogenesis. C3H10T1/2 cells infected with either control or COMP expressing retrovirus were cultured in micromass, then treated with or without BMP-2 (100 ng/ml) as described earlier. (A) Alcian Blue staining. The micromass cultures were fixed on Day 10 and stained with Alcian Blue. The data show that COMP expressing cells treated with BMP-2 have a higher level of Alcian Blue staining than the control cultures treated with BMP-2. (B) Quantification of Alcian Blue staining. The micromasses in (A) were scanned digitally and the staining intensity was measured. The levels were set relative to the control (control set equal to 1). As shown, the staining intensity in the COMP expressing cells treated with BMP-2 is significantly greater than the control cells treated with BMP-2. The error bars indicate the standard deviation of the mean. (C) Metabolic sulfate incorporation. The cultures, as in (A), were radiolabeled with $^{35}SO_4$ (5 μCi/ml final concentration) for a 24-h period ending on Days 4, 7 or 10. ^{3}H-leucine at 1 μCi/ml (final concentration) was also added as a control to standardize for differences in protein levels. The cultures were washed, fixed and the amount of radiolabel incorporation was determined. The ratios of $^{35}SO_4$ to ^{3}H-leucine incorporation on Days 4, 7 and 10 reveal that the COMP expressing cells treated with BMP-2 demonstrate a significantly greater sulfate incorporation on Day 10, compared with the control. Symbols: pBABE alone (open square), pBABE+BMP-2 (open diamond), COMP alone (open circle) and COMP+BMP-2 (open triangle). (D) RT-PCR analysis. Total RNA was isolated from the control and COMP micromass cultures (with and without BMP-2 treatment) on Day 6. One microgram of RNA was processed for RT-PCR using primers specific for mouse collagen type II, collagen, aggrecan and β-actin. The products were electrophoresed on agarose gels stained with ethidium bromide. The stained products reveal that BMP-2 stimulation leads to increased expression of aggrecan and collagen type II. The primers for type II collagen differentiate between the 'A' and 'B' forms of the transcript (the 'B' form is cartilage specific). The reaction products, as shown (left side), were electrophoresed on an agarose gel, which was stained with ethidium bromide. The stained gel shows a higher level of expression of both collagen type II transcripts in the BMP-2-treated cultures relative to the levels of β-actin mRNA. However, expression of COMP had only a minor effect on enhancing the expression of these matrix genes. (E) As a control, RNA was isolated from Day 10 micromass cultures (with and without BMP-2 treatment), and processed for RT-PCR for collagen type II. Shown are the 'A' and 'B' forms of type II collagen. The 'B' form is expressed only in the BMP-2-treated micromass condition.

As a control, the cells not treated with BMP-2 (control and COMP) do not show any Alcian Blue staining.

To determine if the cell numbers within the COMP expressing, BMP-2-treated micromass have changed during the late time periods, the number of viable cells were counted, via trypan blue exclusion staining of trypsinized cells. As seen in Fig. 7A, the cell numbers in all the cultures increased with time, with the exception of the COMP expressing cells treated with BMP-2. These cells demonstrate a noticeable drop in cell numbers by Day 14. The level of protein synthesis in these cultures also reflects this. As seen in Fig. 7B, the level of protein synthesis is relatively the same in all the cultures at Day 10, and at Day 16, with the exception of the COMP expressing cells treated with BMP-2. At Day 16, these cells show a significant drop in the amount of protein synthesis. This reduction in protein synthesis correlates well with the reduced cell numbers in these cultures.

We next assessed whether the drop in cell numbers was due to an increase in apoptosis. The percent apoptotic cells in a population can be derived from flow cytometry analysis, which is evident as cells with less than a 2N (or Diploid)

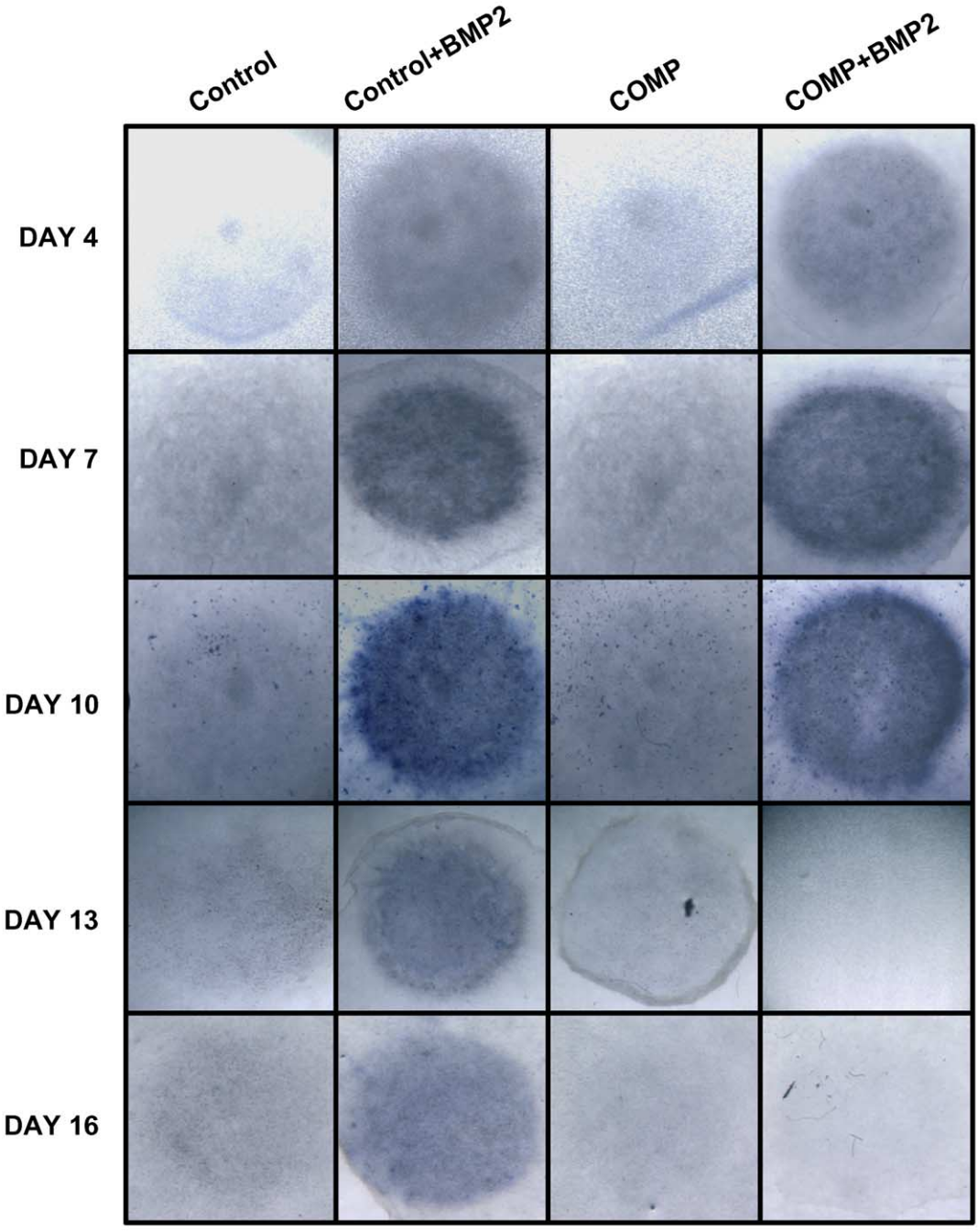


Fig. 6. Loss of Alcian Blue staining in long-term micromass cultures of COMP expressing cells treated with BMP-2. C3H10T1/2 cells infected with either control or COMP expressing retrovirus were plated in micromass cultures, as in Fig. 5, then treated with or without BMP-2 (100 ng/ml). The cultures were fixed and stained with Alcian Blue on the indicated days (4,7,10,13,16). The data show that in the absence of COMP expression, BMP-2 enhances the level of Alcian Blue staining, peaking on Day 10, and remaining above background to Day 16. Following COMP expression by retroviral gene transfer and in the presence of BMP-2, Alcian Blue staining was elevated until Day 10. However, the staining level dropped to background on Days 13 and 16 in the COMP expressing-BMP-2-treated cultures.

DNA content[39]. As seen in Fig. 7C, there is a dramatic increase in apoptosis in the COMP expressing cultures treated with BMP-2 at Day 13. The other culture conditions show a background level of apoptosis that does not appear to increase by Day 13. These data indicate that the loss in cell number in the COMP expressing cells treated with BMP-2 may be due in part to an increase in apoptosis.

While cell number decreases by over fivefold in the COMP expressing micromass cultures treated with BMP-2, we were interested in assessing whether the remaining cells had been altered in terms of their differentiation program. RT-PCR was performed on total RNA, using primers specific for aggrecan, collagen type II and collagen type X. As seen in Fig. 8A, the COMP expressing cells

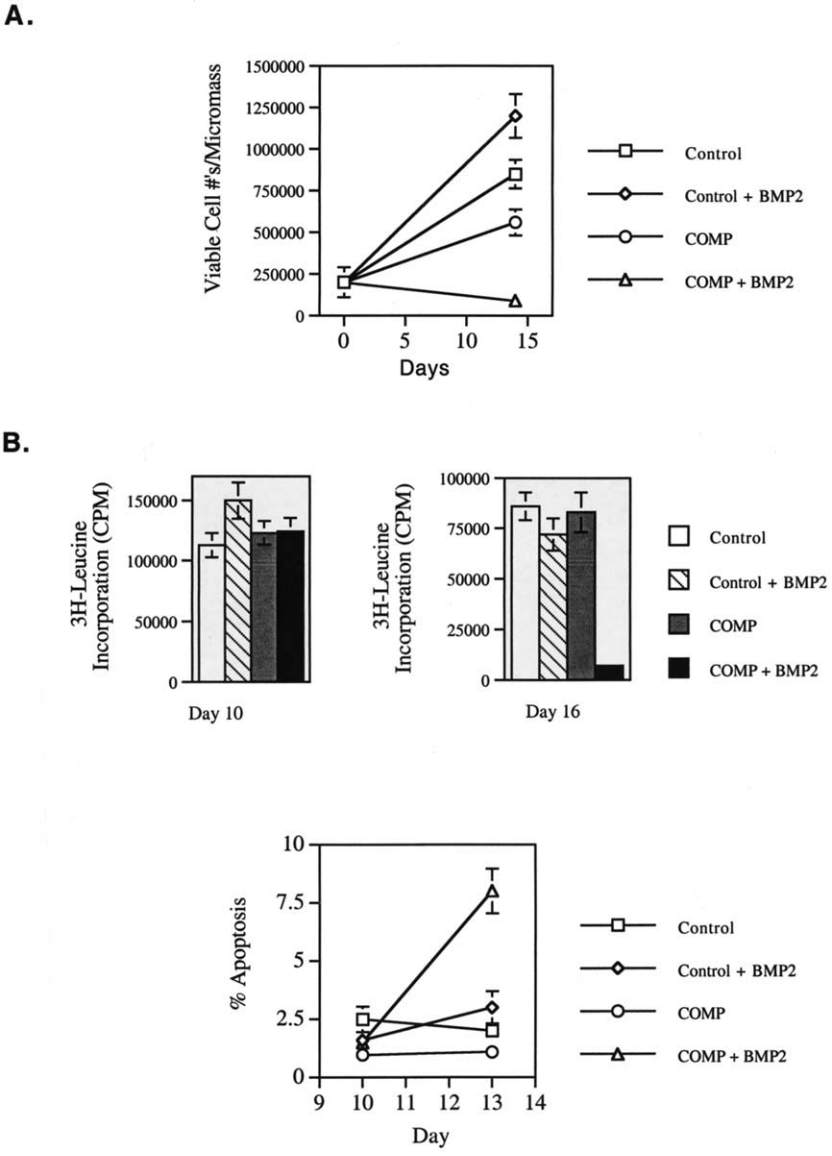
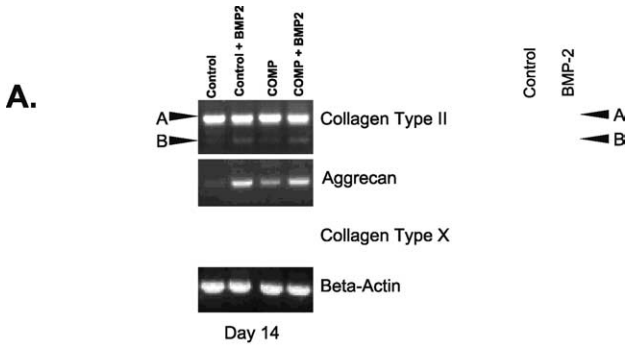


Fig. 7. Reduction in cell numbers and protein synthesis but an increase in apoptosis in long-term micromass cultures of COMP expressing cells treated with BMP-2. C3H10T1/2 cells infected with either control or COMP expressing retrovirus were cultured in micromass, then treated with or without BMP-2 (100 ng/ml). (A) Viable cell counts. The cultures were processed for viable cell counts on Days 0 and 14. The numbers of viable cells are shown, plus or minus the standard deviation from the mean. The data indicate that the COMP expressing cells treated with BMP-2 show a significant decrease in cell numbers on Day 14 compared with the other conditions. (B) ^{3}H-leucine incorporation. ^{3}H-leucine was added to the micromass cultures for a 24-h period ending at Day 10 or 16 (1 μCi/ml final concentration). The counts per minute (CPM) of ^{3}H-leucine incorporation on Day 10 (left side) and Day 16 (right side) are shown, plus or minus the standard deviation of the mean. The data show that the level of leucine incorporation between the different conditions is roughly equal on Day 10, while on Day 16 the COMP expressing cells treated with BMP-2 show a significant decrease in incorporation. (C) Apoptosis. The cultures on Days 10 and 13 were processed for flow cytometry. The percent apoptotic cells was determined as those cells having less than 2N DNA content and is shown plus or minus the standard deviation of the mean. The data show that the level of apoptosis is roughly equal on Day 10 while on Day 13, the COMP expressing cells treated with BMP-2 show a significant increase in the level of apoptosis.

treated with BMP-2 retained their expression of aggrecan and collagen type II. In the cells expressing COMP, but not treated with BMP-2, the levels of aggrecan were slightly enhanced over that of the control. These data indicate that even though there is significant loss of cells in the 'COMP+BMP-2' condition, the remaining cells have retained their chondrogenic differentiation program, as reflected by enhanced expression of aggrecan and collagen type II.

Also assessed by RT-PCR was collagen type X gene expression. This collagen is normally expressed in chondrocytes undergoing hypertrophy[51–53]. As seen in Fig. 8A (left side), collagen type X expression was enhanced in both the BMP-2 and COMP cultures. These data would indicate that at this late stage of culture, both BMP-2 and COMP have a positive effect on the expression levels of collagen type X. As a control, the right hand portion of Fig. 8A, shows both the A and B forms of collagen type II



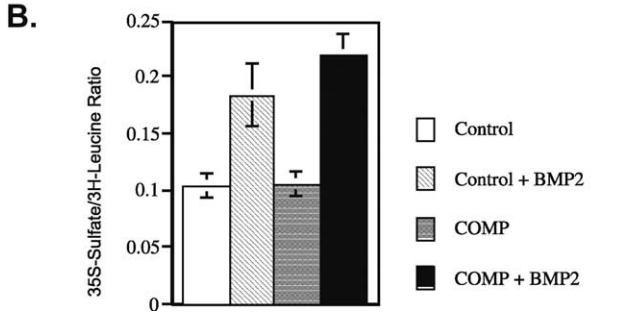


Fig. 8. Markers of chondrogenesis and sulfate incorporation are maintained in long-term micromass cultures of COMP expressing cells treated with BMP-2. C3H10T1/2 cells infected with either control or COMP expressing retrovirus were cultured in micromass, and then treated with or without BMP-2 (100 ng/ml). (A) RT-PCR analysis. (Left side) Total RNA was isolated from the control and COMP expressing micromass cultures (with and without BMP-2 treatment) on Day 14. One microgram of RNA was processed for RT-PCR using primers specific for mouse collagen type II, aggrecan, collagen X and β-actin. The primers for type II collagen differentiate between the 'A' and 'B' forms of the transcript (the 'B' form is cartilage specific). The products were electrophoresed on agarose gels, which were subsequently stained with ethidium bromide. The stained products are shown. The data show that in the cells remaining in the COMP+BMP-2 conditions, expression of aggrecan as well as collagen type II are elevated compared with the controls. (Right side) As a control, RNA was isolated from Day 10 micromass cultures (with and without BMP-2 treatment), and processed for RT-PCR for collagen type II. Shown are the 'A' and 'B' forms of type II collagen. The 'B' form is expressed only in the BMP-2-treated micromass condition. (B) Metabolic sulfate incorporation. $^{35}SO_4$ was added to the culture (5 μCi/ml final concentration) for a 24-h period ending at Day 16 (^{3}H-leucine at 1 μCi/ml final concentration was also added as a control to standardize for differences in protein levels). The ratios of $^{35}SO_4$ to ^{3}H-leucine incorporation on Day 16 are shown. The error bars represent the standard deviation of the mean. The data reveal that the COMP expressing cells treated with BMP-2 demonstrate a significantly enhanced incorporation of sulfate relative to the negative controls.

elevated at a Day 10 time point in the BMP-2-treated micromass cultures.

As an additional test to determine if the chondrogenic phenotype of the COMP expressing cells treated with BMP-2 was retained, the cells were assessed for incorporation of $^{35}SO_4$. As seen in Fig. 8B, the ratio of $^{35}SO_4$ to ^{3}H-leucine incorporation (i.e. $^{35}SO_4$ incorporation normalized to equal levels of protein synthesis) is higher in the BMP-2-treated cultures than in the control and COMP expressing cultures at the Day 16 time point. That this ratio is elevated for the COMP expressing cells treated with BMP-2, indicates that the remaining viable cells in the micromass environment are indeed undergoing an increased level of chondrogenesis, and is consistent with the RT-PCR data presented in Fig. 8A.

Discussion

A central question surrounding cartilage development and function is the role that specific extracellular matrix proteins play in this process. Towards answering this question, we have explored the role of COMP in mesenchymal chondrogenesis. In this study, we have used an *in vitro* system consisting of a murine mesenchymal cell line, C3H10T1/2 cells, which are multipotential and capable of differentiating along chondrogenic lines in a micromass culture system, following treatment with BMP-2[20–22,24]. From the data presented earlier, it appears that COMP has multiple effects on these mesenchymal cells in this *in vitro* system. While it affects cell proliferation and enhances chondrogenic gene expression, it also induces cell death in the presence of BMP-2.

The first function of COMP, apparent from the studies here, is that it affects the level of cell proliferation. It seems that COMP expression slows the rate of growth, without any obvious detrimental effects on cell viability or morphology. This reduction in growth rate corresponds to an increase in the percent of cells in G1 phase and a decrease in the percent of cells in S, G2 and M phases. Since the primary effect of COMP appears to be a partial G1 arrest, this would suggest the involvement of particular cell cycle regulators, namely members of the retinoblastoma gene family as well as the cyclin dependent kinase inhibitors, on COMP-mediated inhibition of growth. These proteins together regulate transit through the G1 checkpoint and are thought to be primarily responsible for induction of G1 arrest[40,41]. An activation of any of these cell cycle regulators by COMP may not be unexpected since it has been demonstrated, for example, that fibrillar collagen type I can inhibit proliferation of arterial smooth muscle cells through the upregulation of the p21/p27 CDIs[42]. While the effect of collagen type I on smooth muscle appears to be mediated through the integrins, it remains to be seen if COMP accomplishes this inhibition through a similar mechanism.

From the data here, it appears that COMP also has an effect on enhancing some aspects of chondrogenesis. This enhancement occurs during the middle phase of the micromass culture period (i.e. by Day 10) and only in the presence of BMP-2 treatment. This suggests a number of features for COMP function. One is that COMP mediates a signaling pathway in the cell and that this pathway converges with that of BMP-2 signaling. Since COMP is part of the thrombospondin family, which binds the CD36 and CD47 receptors[43,44], the effects of COMP may be mediated through a thrombospondin-type receptor signaling mechanism. In addition, given that COMP contains an RGD sequence, it is possible that COMP mediates signaling through an integrin. The other important feature of the COMP enhancement of BMP-2 induced chondrogenesis is that BMP-2 and COMP function in a synergistic way, suggesting that the signaling pathway of COMP may converge on that of BMP-2. As mentioned earlier, enhancement of chondrogenesis by COMP, in the presence of

BMP-2, appears to require about 7–10 days of culture to be evident. This temporal requirement is of potential significance. From the data presented here and elsewhere[20], BMP-2 acting alone as a differentiation-inducing signal on C3H10T1/2 micromass cultures requires a 3–5 day treatment period during the initial phase of culture. Since the effect of COMP on chondrogenesis requires 10 days, COMP signaling is likely to act downstream of BMP-2 signaling.

An interesting outcome of the studies presented here was that ectopic expression of COMP had a pro-apoptotic effect on the cell cultures. The data showed that COMP increases the extent of apoptosis late in the culture period (i.e. after Day 10), but only in the presence of BMP-2. This corresponded to a loss of cells in the micromass culture along with a loss of Alcian Blue staining intensity. At this time we do not know if this is a direct or indirect effect of COMP, which remains to be determined. Surprisingly, the remaining cells in the micromass retained their differentiated state, as reflected by elevated aggrecan and collagen type II gene expression and sulfate incorporation. Thus, BMP-2 and COMP signaling have a rather unique synergistic effect late in the culture period, corresponding to a fully chondrocytic cellular phenotype. That this effect of COMP only occurs in the presence of BMP-2, suggests that an apoptotic mechanism is induced by the convergence of COMP and BMP-2 signaling.

A number of possibilities exist for these cells to be induced to undergo apoptosis by BMP-2 treatment and ectopic expression of COMP. The first is that the maturation pathway of chondrocytes during endochondral ossification is well known to result in induction of apoptosis[54]. Although we do not have solid morphological evidence that BMP-2-treated C3H10T1/2 micromass cultures are undergoing hypertrophy (i.e. the cells are enlarged), we surprisingly do see induction of collagen type X by both COMP and BMP-2. Collagen type X is well known as a very specific marker of hypertrophic chondrocytes[51–53]. That collagen type X is induced in these cells is in line with our finding that COMP is expressed at high levels in regions undergoing both hypertrophy and mineralization during development. This can be seen in Fig. 1, where the high level of COMP expression in the digits and tail vertebra overlaps with the pink Mallory's stain, the latter indicating formation of mineralized bone in the region of chondrocyte hypertrophy. It is therefore possible that COMP and BMP-2 aid in the induction of apoptosis through a chondrocyte maturation pathway. In this regard it is known that BMP signaling (in particular BMP-6, 2 and 7) plays a very important role in the induction of hypertrophy[54,55]. Thus, it may be that during development, or during fracture repair in the adult, BMP and COMP combine to facilitate chondrocyte maturation through hypertrophy and apoptosis, as part of endochondral ossification. However, additional work will be needed to determine if this is indeed the case.

An additional possibility for the induction of apoptosis by COMP and BMP-2 involves interdigital apoptosis. Given that the BMP-2 effect on enhancement of chondrogenesis is an early event, the cells that respond to COMP appear committed to the chondrocytic phenotype. Along these lines, the patterning of the cartilaginous model requires spatio-temporal specific apoptosis that can be observed in the interdigital zones of the developing limb[45]. As shown in Fig. 1, COMP expression is high in the digital zones of the limb (i.e. in the footpad), immediately adjacent to the areas that are undergoing cell death. It has been previously shown that BMP-2, while chondrogenic for the undifferentiated mesenchyme, is responsible for apoptosis in the interdigital zones[46–49]. By extrapolating to the *in vitro* micromass cultures that ectopically express COMP and are treated with BMP-2, we would expect enhanced apoptosis in a significant portion of the cells, while the remaining viable cells would be chondrocytic. We have observed here that early chondrogenesis is enhanced, yet later in culture we see an induction of apoptosis.

In conclusion, we have identified a novel role for COMP in chondrogenesis. COMP enhances BMP-2-mediated chondrogenesis during the middle phase of culture and then leads to enhanced apoptosis late in culture. Use of this *in vitro* micromass culture system should enable us to further probe the mechanisms responsible for these effects of COMP and their relation to chondrocyte differentiation and cartilage biology. These results are especially pertinent given the recent findings that COMP null mice demonstrate no detectable skeletal defects, including effects on cartilage[50]. It therefore may be that the *in vitro* mechanism used here, in conjunction with a gain-of-function approach of retroviral gene transfer, may be optimal for assessing the exact role of COMP in chondrogenesis.

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