# Regulation of Articular Chondrocyte Phenotype by Bone Morphogenetic Protein 7, Interleukin 1, and Cellular Context Is Dependent on the Cytoskeleton

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**Bone morphogenetic proteins (BMPs) induce cartilage differentiation and morphogenesis. There are profound changes in the cytoskeletal architecture during the morphogenesis of cartilage. To investigate the possibility that morphogenetic signals such as BMPs may regulate chondrocyte phenotype by modulation of cytoskeletal protein expression, we determined whether the expression and distribution of cytoskeletal proteins in chondrocytes are regulated by bone morphogenetic protein 7 (BMP 7), interleukin 1 (IL-1), and cellular context. Addition of BMP 7, a morphogen that induces chondrogenesis, to primary cultures of bovine and murine chondrocytes induced increased expression of four cytoskeletal proteins: tensin, talin, paxillin, and focal adhesion kinase (FAK). The expression of cytoskeletal proteins is dependent on cellular context; compared to monolayer, chondrocytes in suspension exhibited increased expression of cytoskeletal components. Conversely, addition of IL-1, a catabolic cytokine, induced loss of chondrocyte phenotype and decreased the expression of these cytoskeletal components. Treatment of chondrocytes with cytochalasin D (an agent that disrupts the actin cytoskeleton) inhibited BMP 7-induced upregulation of tensin, talin, paxillin, and FAK, and blocked the effect of BMP 7 on chondrocyte phenotype. Taken together these data demonstrate that cytoskeletal components play a critical role in the response to morphogens and cytokines in the regulation of chondrocyte phenotype.** © 2001 Elsevier Science

***Key Words:* tensin; talin; paxillin; focal adhesion kinase; collagen type II.**

## INTRODUCTION

The adhesive interactions between a cell and its surrounding extracellular matrix (ECM)[2] regulate cell phenotype, migration, growth, differentiation, and morphogenesis [1, 2]. In cartilage, a tissue that has a high extracellular matrix-to-cell ratio, matrix–cell interactions are particularly important mediators of these processes. In most cell types focal adhesions are critical for cell–matrix interactions; focal adhesion junctions mediate the linkage of extracellular matrix to the cytoskeleton via a supramolecular complex of transmembrane (integrins) and cytoplasmic proteins (reviews; [3, 4]). This linkage allows the cell to respond rapidly to dynamic changes in both the extracellular and the intracellular environment, providing a connection between extracellular cues and the nuclear genome. Changes in the cellular environment may affect focal adhesion number and distribution and therefore organization of the actin cytoskeleton, influencing gene expression [5]. Several of the cytoplasmic proteins associated with focal adhesion complexes are involved in signal transduction of morphogens and cytokines [6–11].

The effects of cell shape and actin organization on chondrocyte phenotype is well known [12–19]. Benya and Shaffer (1982) demonstrated that when chondrocytes are maintained in a flattened configuration in monolayer culture, the chondrocytes lose their differentiated phenotype. The chondrocyte phenotype can be recovered if these same cells are then maintained in a rounded configuration in agarose culture. Brown and Benya (1988) demonstrated that the effect of cell shape on chondrocyte phenotype was indirect, and that changes in organization of the actin cytoskeleton were in fact responsible for subsequent changes in chondro-

[2] Abbreviations used: BMP, bone morphogenetic protein; CT, cycle threshold; ECL, enhanced chemiluminescence; ECM, extracellular matrix; FAK, focal adhesion kinase; IL-1, interleukin-1; RT-PCR, reverse transcription–polymerase chain reaction.

cyte phenotype. Further studies have confirmed the importance of actin organization in control of chondrocyte phenotype; however, to date, a role for focal adhesion proteins in governing actin organization in chondrocytes and thereby chondrocyte phenotype has not been systematically investigated. As mentioned, focal adhesion protein expression and distribution can influence organization of the cytoskeleton and thereby influence phenotype. Many focal adhesion proteins are also involved in signal transduction pathways, so may be able to influence phenotype independently. A number of integrin subunits and cytoplasmic proteins associated with focal adhesions have been described within chondrocytes [20–22].

Hormones, growth factors, and cytokines influence chondrocyte phenotype. To date, the effect of these factors on chondrocyte cytoskeleton has not been investigated. Bone morphogenetic proteins (BMPs) influence the phenotype, growth, and differentiation of chondrocytes. BMPs are a family of growth and differentiation factors that are key regulators of the formation and homeostasis of cartilage and bone [23–24]. Over 15 have been cloned and expressed in humans and mice. BMPs signal via membrane-bound serine/threonine kinase receptors [25]. Binding of BMP to its receptor initiates phosphorylation of Smad proteins and the formation of a Smad complex. This complex is able to translocate into the nucleus and activate transcription machinery for BMP-response genes [26]. BMPs also signal via mitogen-activated protein kinases [27]. Interleukin 1 (IL-1) can also influence chondrocyte phenotype. IL-1 was originally defined as a molecule synthesized by monocytes and macrophages. It is now known that a number of nonimmune cells synthesize IL-1, including chondrocytes. In cartilage, IL-1 is able to control the synthesis and activation of a number of proteinases involved in cartilage destruction, e.g., stomelysin and collagenase [28, 29]. IL-1 is also able to suppress the synthesis of type II collagen, a key component of articular cartilage [30].

To determine whether the effects of environmental cues such as cellular context, BMP 7, and IL-1 on chondrocyte phenotype are facilitated by components of the cytoskeleton, we have investigated the effect of these signals on the expression and distribution of four key cytoskeletal components: tensin, talin, paxillin, and FAK. Changes in chondrocyte phenotype were monitored by type II collagen expression levels. Exposure to factors that promote chondrocyte phenotype, i.e., a rounded configuration and addition of morphogen BMP 7, resulted in increased expression of focal adhesion proteins and a concomitant increase in type II collagen. Exposure to factors that promote loss of chondrocyte phenotype, i.e., a flattened configuration, and addition of catabolic cytokine IL-1 resulted in de-

creased expression of focal adhesion proteins and a concomitant decrease in type II collagen expression. Distribution of the focal adhesion proteins also influenced chondrocyte phenotype. Finally, we have demonstrated that BMP 7-mediated change in chondrocyte phenotype is dependent on components of the cytoskeleton.

## MATERIALS AND METHODS

*Tissue samples.* Bovine articular cartilage was harvested from the metatarsal phalangeal joint of young calves (approximate age 2– 4 weeks). Murine articular cartilage was harvested from the knee joint of newborn C57B mice (Charles River, Boston, MA).

*Antibodies.* Monospecific antibodies to tensin (UC70, [31]) talin (Transduction Lab., Los Angeles, CA, and Santa Cruz Biotech, Santa Cruz, CA), paxillin (Transduction Lab. and Santa Cruz Biotech), and FAK (Transduction Lab. and Santa Cruz Biotech) were used. FITC–phalloidin (Sigma, St. Louis, MO) was used to label the actin cytoskeleton.

*Chondrocyte isolation.* Bovine cartilage was first treated for 20 min with 0.5% pronase at 37°C (in DMEM/F12 containing 5% fetal calf serum) (Sigma) to disrupt the cartilage matrix. Matrix proteolysis was completed using 300 units/ml collagenase type IA (in DMEM/F12 containing 5% fetal calf serum) (Sigma) for 4 h at 37°C. Murine knee joint cartilage was placed into 0.5% trypsin and incubated at 37°C for 20 min with intermittent vortexing. This ensured removal of any attached muscle and tendon. The murine knee joint cartilage matrix was then digested using 300 units/ml collagenase type IA (in DMEM/F12 containing 5% fetal calf serum) (Sigma). Released chondrocytes (both bovine and murine) were passed through a 70-$\mu$m nylon mesh (Falcon, Bedford, MA), washed using phosphate-buffered saline, pH 7.4 (PBS), and then placed into DMEM/F12 medium (GIBCO, Rockville, MD) supplemented with 50 $\mu$g/ml ascorbate phosphate (Sigma), 1% penicillin/streptomycin (GIBCO), and 5% fetal calf serum (GIBCO).

*Cell culture.* Chondrocytes and NIH 3T3 fibroblasts were cultured in DMEM/F12 containing 5% fetal calf serum (GIBCO) and supplemented with 50 $\mu$g/ml ascorbate (Sigma) and 1% penicillin/streptomycin (GIBCO). Cells were maintained in either monolayer culture or suspension culture for approximately 6 days before being used in experimental procedures. Monolayer cultured cells were grown in 100-mm tissue-culture dishes (Falcon). For suspension culture, 100-mm dishes were coated with 3 ml of 1% agarose (Sigma). Prior to addition of either recombinant human BMP 7 (gift from Dr. K. Sampath) or recombinant human IL-1$\beta$ (R & D Systems, Minneapolis, MN), cells were serum-starved for 48 h, and then fed with fresh serum-free medium immediately before the addition of either BMP 7 or IL-1. In some experiments, monolayer cultures were treated with cytochalasin D (3 $\mu$g/ml, Sigma), an agent known to disrupt the actin cytoskeleton [32]. All experiments were performed in triplicate.

*Protein extraction.* Cellular proteins were extracted from monolayer- and suspension-cultured chondrocytes and NIH 3T3 fibroblasts using a 1% Triton X (Sigma) buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2 $\mu$l/ml leupeptin; all from Sigma). Samples were centrifuged at 20,000$g$ to remove cellular debris. DNA content was assessed using PicoGreen Assay (Molecular Probes, Eugene, OR) as an indicator of cell number. Samples, normalized via DNA content, were run on 7% Tris–glycine gels using the discontinuous SDS–PAGE method described by Laemmli [33]. Protein extracted from approximately 150,000 cells was loaded in each lane. Samples were electrophoresed at 125 V in running buffer
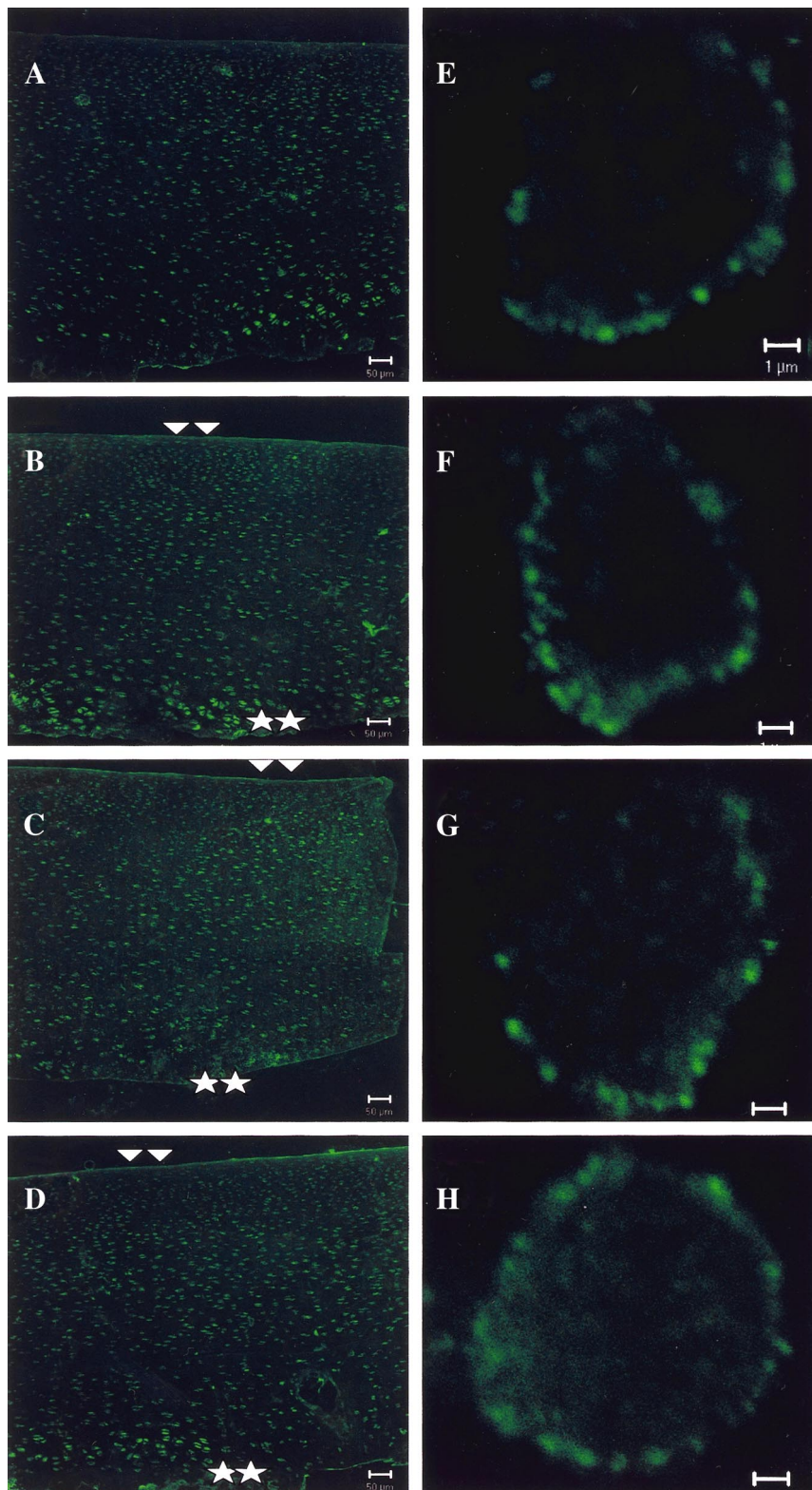
**FIG. 1.** Sections of bovine articular cartilage were immunolabeled with antibodies specific to tensin (A, E), talin (B, F), paxillin (C, G) and FAK (D, H). Labeling was visualized using a Zeiss 510 confocal microscope. Pictures taken at low magnification (scale bar = 50 $\mu$m) demonstrated that (A) tensin, (B) talin, (C) paxillin, (D) FAK were expressed by chondrocytes through the entire depth of the articular cartilage sections. However, labeling appeared less intense in the surface zone (arrowheads) compared to the deep zone (stars) of the tissue. At higher magnification (E–H, scale bar = 1 $\mu$m), it was evident that labeling was punctate and membrane-associated. This result implies that tensin, talin, paxillin, and FAK are participating in focal adhesion junctions within chondrocytes.
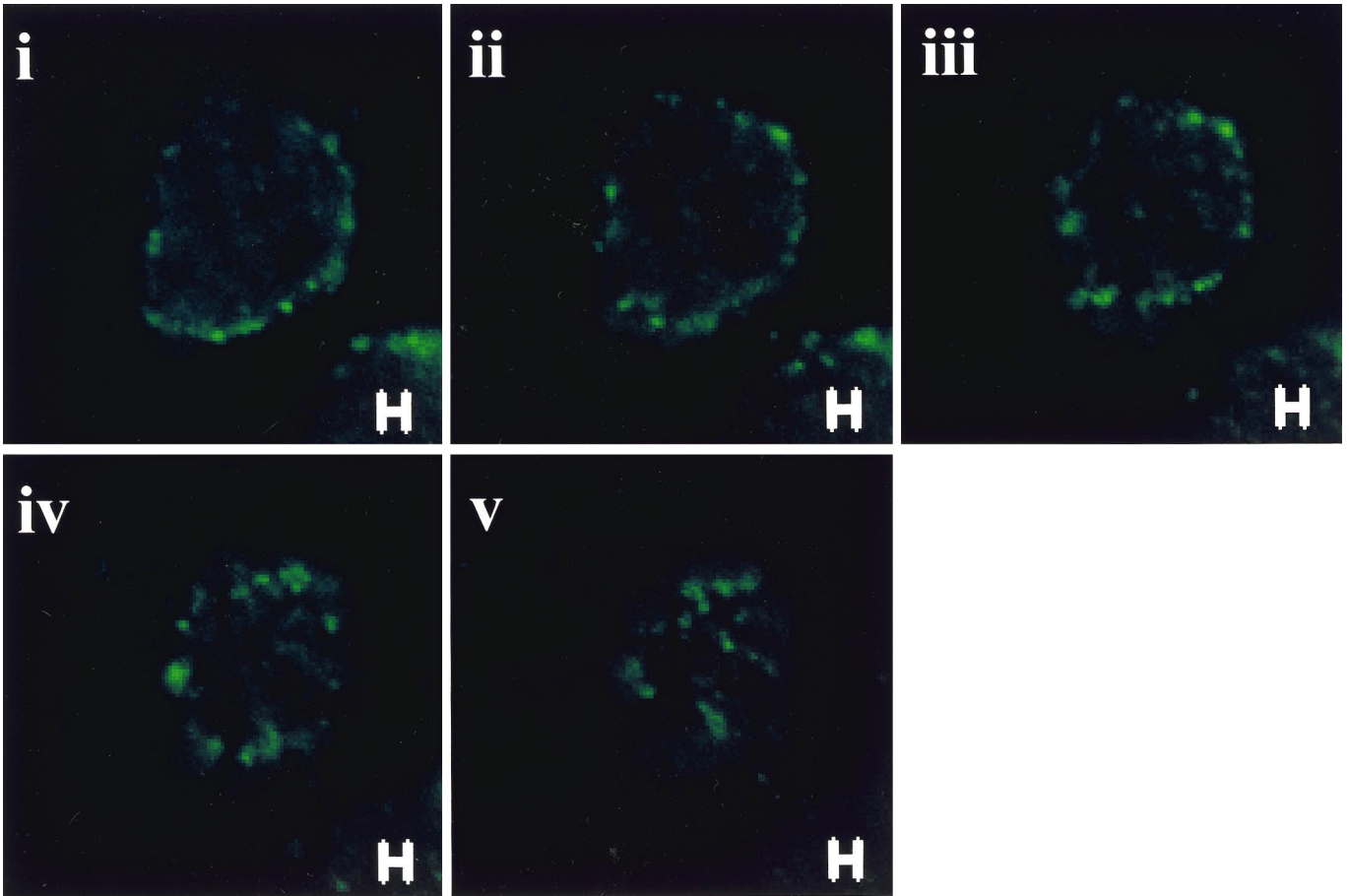
**FIG. 2.** Confocal section series (i–v) demonstrating distribution of tensin within a chondrocyte present in a tissue section (i = center of the chondrocyte, v = bottom of the chondrocyte, i–v represent 1-$\mu$m steps through the chondrocyte) (Scale bar = 1 $\mu$m). Labeling for tensin was punctate and membrane-associated throughout the depth of the chondrocyte. Similar labeling patterns were observed when chondrocytes were labeled using antibodies specific for talin, paxillin, and FAK (data not shown). These data demonstrate that focal adhesion junctions are uniformly distributed over the chondrocyte membrane.

(0.025 M Tris, 0.2 M glycine, 0.1% SDS) and run along side prestained molecular weight markers (Rainbow markers, Amersham–Pharmacia, Piscataway, NJ).

*Western blotting.* Proteins were electrotransferred onto nitrocellulose using a BioRad (Hercules, CA) mini Transblot unit, at a constant current of 0.3 mA for 1 h 30 min in transfer buffer (25 mM Tris, 192 mM glycine (pH 8.3), and 20% methanol). After transfer, the nitrocellulose membranes were blocked with TBS (Tris-buffered saline; 50 mM Tris, 200 mM NaCl) containing 5% dried nonfat milk, 1% goat serum, 1% horse serum (GIBCO) for 1 h. The membranes were then incubated with specific antibodies (polyclonal tensin, 1:2000; monoclonal paxillin, 1:5000; monoclonal FAK, 1:1000; monoclonal talin, 1:1000) diluted in blocking solution for 1 h 30 min. The membranes were washed 3 × 5 min in TBS containing 0.05% Tween (Sigma) (TBST) and then incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, 1:5000; donkey anti-rabbit, 1:3000 (Transduction Lab)) diluted in TBST for 1 h. After washing (3 × 5 min in TBST), the blots were developed using enhanced chemiluminescence (ECL, Amersham–Pharmacia), and bands detected using autoradiography.

*Immunocytochemistry.* Full-depth cubes of bovine cartilage (5 mm²) and entire mouse knee joints were fixed in 4% paraformaldehyde (Sigma) overnight. Following washing with PBS, tissues were

placed in 5% sucrose (Sigma) overnight and 8-$\mu$m cryosections made using a Bright (Fairfield, NJ) cryomicrotome. Cultured chondrocytes were washed using PBS, fixed briefly in 4% paraformaldehyde, and then washed again using PBS. In some cases tissue sections and cells were permeabilized using 0.01% Triton in PBS (pH 7.4) and incubated with fluorescein-conjugated phalloidin for 1 h prior to fixation. Both tissue sections and cells were blocked using 1:20 goat serum (Sigma), incubated with primary antibodies (tensin, 1:100; talin, 1:100; paxillin, 1:500; FAK, 1:100) for 1 h, washed using PBS and then incubated with fluorescein-conjugated secondary antibodies (1:80 (Sigma)) for 1 h. For dual-label studies, tissue sections and cells were incubated for 1 h with a combination of tensin (polyclonal) antibody and antibodies to talin, paxillin, or FAK. The tissue sections and cells were washed and then incubated for 1 h with a combination of Texas Red-conjugated goat anti-rabbit secondary antibody (1:200 (Molecular Probes)) and fluoroscein-conjugated goat anti-mouse secondary antibody. Following washing using PBS, tissue sections and cells were mounted in VectorMount (Vector, Burlingame, CA). Fluorescent labeling was visualized using a Zeiss 510 confocal microscope.

*RNA isolation and quantitative RT-PCR.* RNA was isolated from cultured bovine chondrocytes using the Qiagen RNeasy kit (Qiagen, Valencia, CA). To ensure the complete removal of DNA we included

a 15-min DNase I treatment prior to the wash step of the isolation (Qiagen). Quantitative RT-PCR (TaqMan, Applied BioSystems, Foster City, CA) was used to determine expression levels of type II collagen in the samples. Oligonucleotide primers and a reporter probe corresponding to cDNA sequences for type II collagen (primer 1: TGGAAAGGCCATCACCATCT; primer 2: CTCAGCACCAGCATCACCC; probe: CCTCGCTCTAGGACGGTTGTAGTTCA) were designed using Primer Express software (Applied BioSystems). Primers (18S) and reporter probe (Applied BioSystems) were used as a control for the amount of RNA in each sample. Quantitative RT-PCR was performed using the TaqMan EZ RT-PCR core kit and the ABI Prism 7700 sequence detector (Applied BioSystems). Thermal cycling conditions were 2 min at 50°C, 30 min at 60°C, 5 min at 60°C, and 40 cycles of 20 s at 94°C, 1 min at 62°C. Each sample was analyzed in triplicate. The average CT value of each sample (CT value represents the cycle at which a statistically significant increase in signal magnitude, i.e., amplified PCR product, is detected) was compared between experimental groups, and the results were documented as fold differences in expression level of type II collagen.

## RESULTS

### Localization of Tensin, Talin, Paxillin, and FAK in Cartilage and Cultured Chondrocytes

Tensin, talin, paxillin, and FAK were localized within chondrocytes throughout the tissue depth of bovine articular cartilage (Figs. 1A–1D). However, labeling for tensin, talin, paxillin, and FAK appeared to be more intense within the rounded chondrocytes present in the deep zone (the area adjacent to the subchondral bone) compared to labeling seen within the flattened chondrocytes present in the articular surface of the tissue. Labeling for these molecules was mainly punctate and membrane-associated (Figs. 1E–1H and Fig. 2), suggesting that these molecules participate in focal adhesion complexes within chondrocytes. A small amount of diffuse, cytoplasmic labeling was observed in some cells, indicating that a cytoplasmic pool of these molecules exists in chondrocytes. The combined data suggest that rounded chondrocytes in the deep zone of articular cartilage may express more focal adhesions than flattened chondrocytes in the surface zone of the tissue.

Cultured chondrocytes were also immunopositive for tensin, talin, paxillin, and FAK (Figs. 3A–3H). Labeling of chondrocytes for tensin, talin, paxillin and FAK was punctate and membrane-associated, suggesting that cultured chondrocytes are also able to form focal adhesion complexes. Interestingly, the "type" of punctate labeling observed in monolayer and suspension cultured chondrocytes was very different. In monolayer culture (Figs. 3A–3D) large areas of punctate membrane-associated labeling were observed. Labeling appeared to be polarized and streaks of punctate labeling for tensin, talin, paxillin, and FAK appeared to align to the extended edges of the cells. Punctate labeling for tensin, talin, paxillin, and FAK was also observed in suspension cultures of chondrocytes (Figs. 3E–3H). The areas of punctate labeling were much smaller than those observed in monolayer culture; however, the number of labeled areas was much greater. The labeling pattern of tensin, talin, paxillin, and FAK observed in suspension-cultured chondrocytes was comparable to that observed in *ex vivo* tissue sections (Figs. 1E–1H). More cytoplasmic labeling was observed within suspension-cultured chondrocytes, suggesting that turnover of these molecules may be higher in rounded chondrocytes. The combined data suggest that focal adhesions differ in terms of both size and distribution in monolayer- and suspension-cultured chondrocytes.

The organization of the actin cytoskeleton in suspension- and monolayer-cultured chondrocytes was also very different (Figs. 4A and 4B). In suspension culture (Fig. 4A) the actin cytoskeleton consisted of an intricate network of fine actin fibers that made numerous connections to the plasma membrane. In monolayer culture (Fig. 4B) the actin cytoskeleton consisted of thick struts of actin extended from across the length of the chondrocyte.

### Tensin Colocalizes with Talin, Paxillin, and FAK in Cultured Chondrocytes

Dual-labeling studies revealed that tensin colocalizes with talin (Figs. 5A–5F), paxillin, and FAK (data not shown) in monolayer- and suspension-cultured chondrocytes. These data provide further evidence that focal adhesion junctions exist in chondrocytes grown in culture.

### Chondrocytes in Suspension Culture Express More Tensin, Talin, Paxillin, and FAK Than Monolayer-Cultured Chondrocytes (Cell Context Influences the Expression of Focal Adhesion Proteins)

Sequential protein extraction with buffers of increasing extractability demonstrated that a 1% Triton X-based extraction buffer was sufficient to fully extract tensin, talin, paxillin, and FAK from both suspension- and monolayer-cultured chondrocytes. Use of a 0.1% Triton-based extraction buffer resulted in extraction of a cytoplasmic pool of free tensin, talin, paxillin, and FAK that is not involved in the supramolecular focal adhesion complex. The existence of a cytoplasmic pool of tensin, talin, paxillin, and FAK in chondrocytes is supported by our immunocytochemical observation that cultured chondrocytes displayed some cytoplasmic labeling that was not associated with the plasma membrane (Fig. 3).

Chondrocytes grown in suspension culture expressed high levels of tensin, talin, paxillin, and FAK compared to chondrocytes grown in monolayer culture (Fig. 6A). In contrast, NIH 3T3 fibroblasts synthesized little or no tensin, talin, paxillin, and FAK when grown in suspension culture (Fig. 6B), compared to monolayer cultures.

## Chondrocyte Phenotype Is Dependent on Cellular Context

Quantitative PCR (TaqMan) (Fig. 6C) was used to quantitate the relative expression of type II collagen mRNA present in RNA isolated from both suspension- and monolayer-cultured chondrocytes. Type II collagen is a characteristic marker of chondrocyte phenotype and is downregulated when chondrocytes begin to dedifferentiate [34]. The data demonstrate that in suspension culture chondrocytes express significantly more type II collagen mRNA than in monolayer cultures. The decrease in type II collagen expression observed in monolayer culture was concomitant with decreased expression of tensin, talin, paxillin, and FAK in monolayer culture (Fig. 6A). The increase in type II collagen expression observed in suspension culture was concomitant with increased expression of tensin, talin, paxillin, and FAK in suspension culture (Fig. 6A). From these data we concluded that expression of tensin, talin, paxillin, and FAK and expression of type II collagen, i.e., chondrocyte phenotype, are linked. Next we performed experiments to further investigate the relationship between expression of focal adhesion molecules and chondrocyte phenotype.

## BMP 7 Upregulates the Expression of Tensin, Talin, Paxillin, and FAK and Promotes Chondrocyte Differentiation

We determined that addition of 50 ng/ml BMP 7, a morphogen known to induce chondrocyte phenotype [35], to monolayer cultures over a 24-h time period led to maximal expression of tensin, talin, paxillin, and FAK (Figs. 7A and 7B). Addition of BMP 7 to monolayer cultures resulted in a significant increase in the expression of tensin, talin, paxillin, and FAK compared to control cultures (Figs. 7A, 7B and 8A), and a concomitant increase in type II collagen expression (Fig. 8B). On the other hand, BMP 7 had little effect on the expression of tensin, talin, paxillin, and FAK or of type II collagen by suspension-cultured chondrocytes (Figs. 8A and 8B). It is likely that there is maximal expression of cytoskeletal proteins and type II collagen when chondrocytes are grown in suspension culture. These
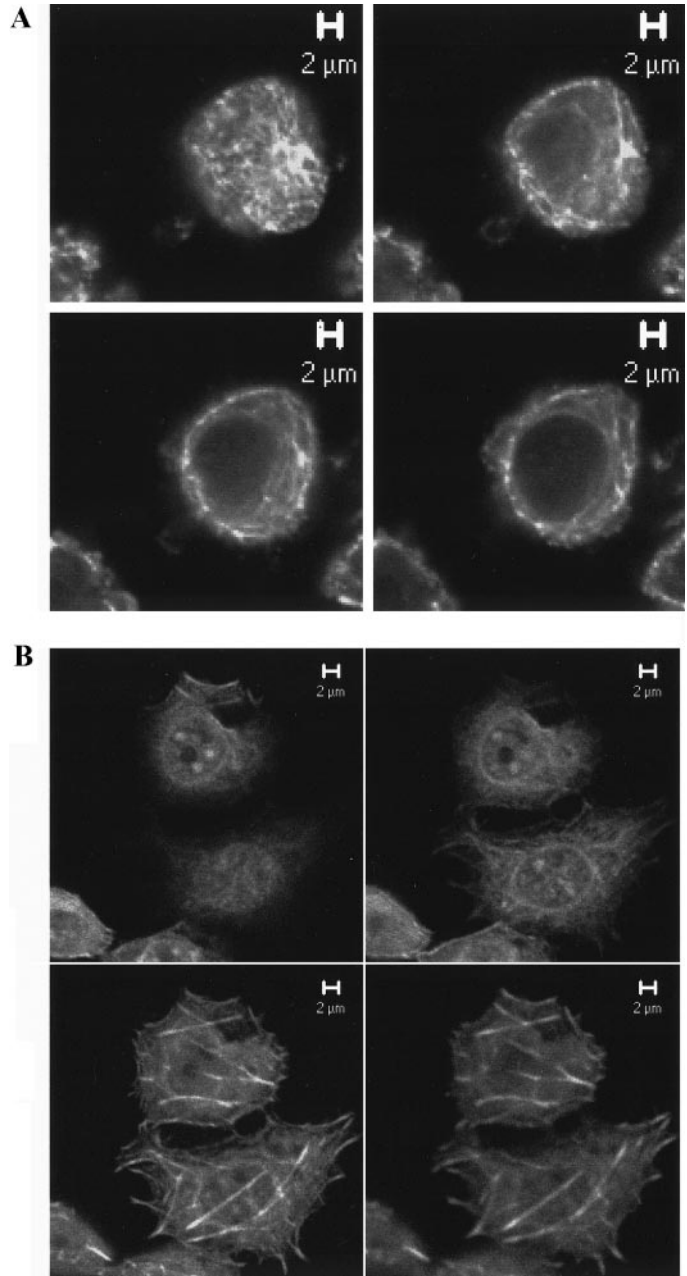


**FIG. 4.** Confocal section series of cultured chondrocytes stained with FITC–phalloidin (Scale bar = 2 $\mu$m). The organization of the actin cytoskeleton in chondrocytes grown in suspension culture (A, i–iv) and monolayer culture (B, i–iv) is very different. In suspension culture (A, i–iv) the actin cytoskeleton consists of an intricate network of fine actin fibers that make numerous connections to the plasma membrane. In monolayer culture (B, i–iv) thick struts of actin extend across the length of the chondrocyte. The first section (i) in each series represents the top of the chondrocyte; i–iv represent 1-$\mu$m steps through the chondrocyte.

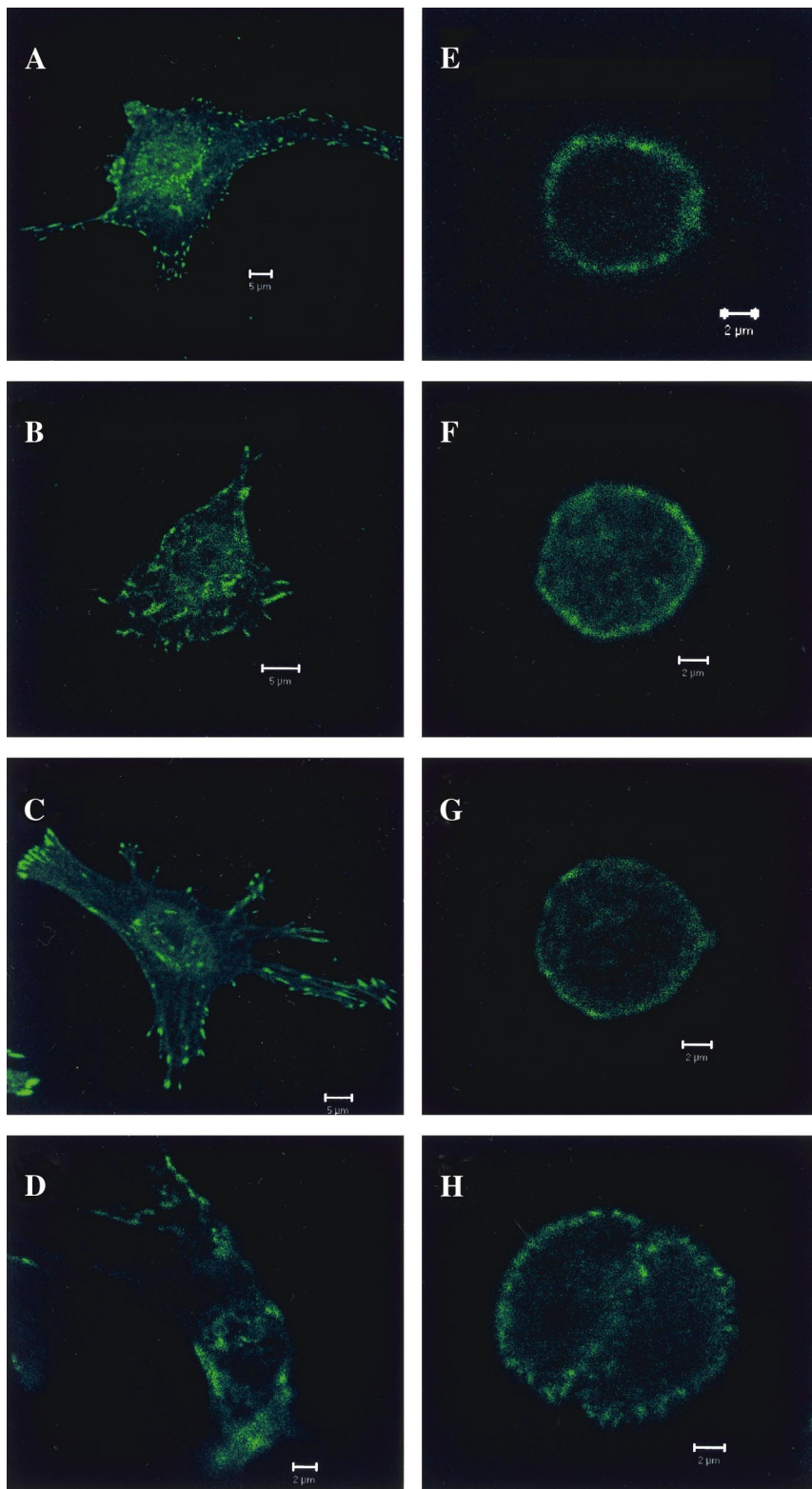data support our hypothesis that expression of focal adhesion proteins and chondrocyte phenotype are linked.

**FIG. 3.** Bovine chondrocytes grown in monolayer culture (A–D, scale bar = 5 $\mu$m) and suspension culture (E–H, scale bar = 2 $\mu$m) were labeled using specific antibodies for tensin (A, E), talin (B, F), paxillin (C, G), and FAK (D, H). Labeling for all four focal adhesion proteins in chondrocytes grown in both monolayer (A–D) and suspension (E–H) culture was punctate and membrane-associated, strongly suggesting that focal adhesion junctions are present within these cells. The focal adhesion junctions localized in chondrocytes grown in monolayer culture (A–D) appeared to be larger than those localized in suspension-cultured chondrocytes (E–H). More, but smaller, focal adhesion junctions were localized in chondrocytes grown in suspension culture.
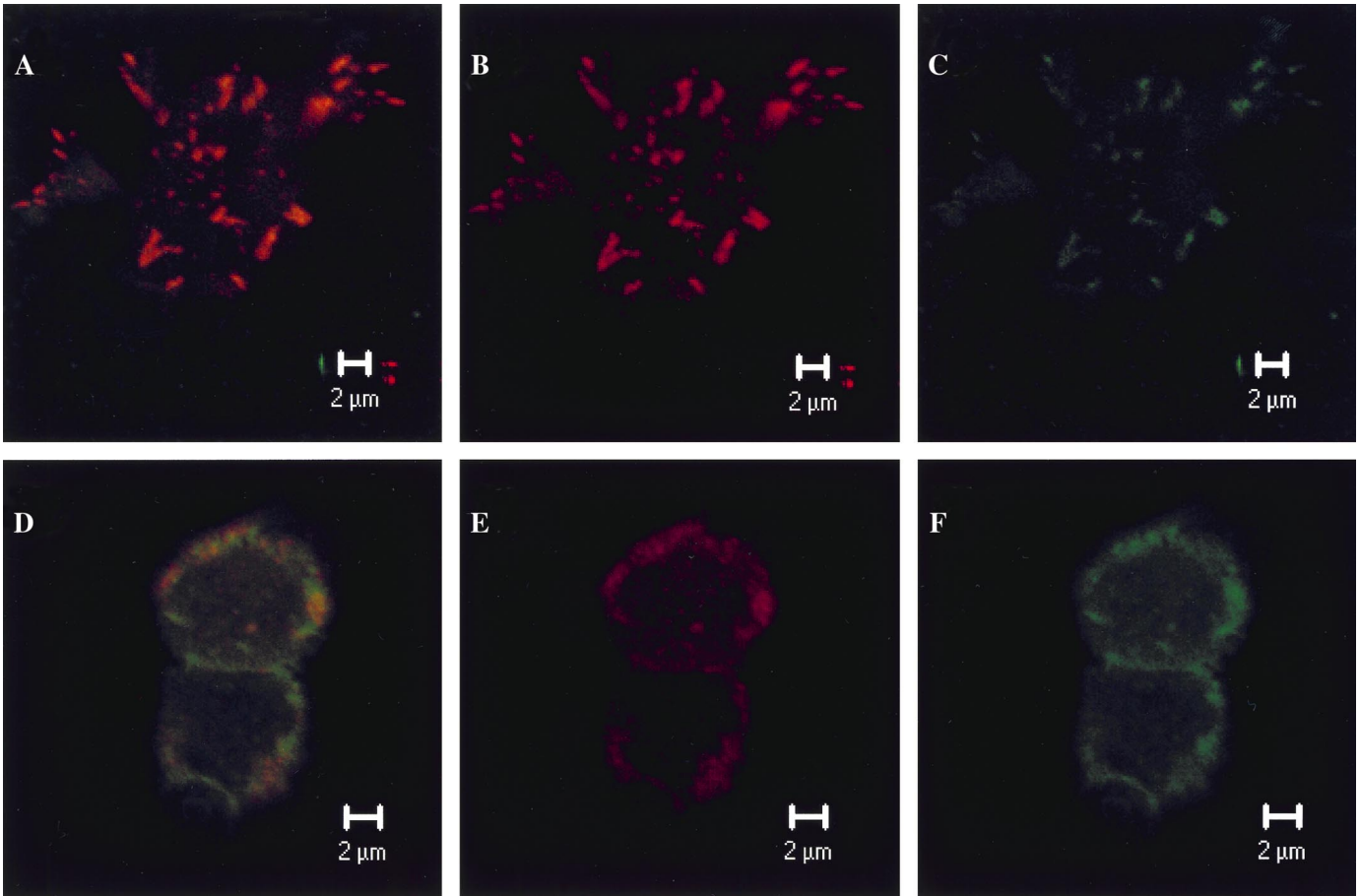
**FIG. 5.** Chondrocytes grown in monolayer culture (A–C, scale bar = 2 $\mu$m) and chondrocytes grown in suspension culture (D–F, scale bar = 2 $\mu$m) were colabeled for paxillin (green) and tensin (red). (A, D) Double label. Areas of orange labeling represent colocalization of tensin and paxillin. (B, E) Red channel only (i.e., tensin label). (C, F) Green channel only (i.e., paxillin label). These data strongly suggest that focal adhesion junctions are present in chondrocytes grown in both monolayer and suspension culture.

### IL-1 Downregulates the Expression of Tensin, Talin, Paxillin, and FAK and Promotes Chondrocyte Dedifferentiation

Addition of IL-1, a catabolic cytokine associated with loss of chondrocyte phenotype [30, 36], to chondrocytes in suspension culture resulted in a decrease in tensin, talin, paxillin, and FAK expression (Fig. 9A) and a concomitant decrease in type II collagen expression (Fig. 9B). Again, these data support our hypothesis that focal adhesion molecule expression and chondrocyte phenotype are linked. The effect of IL-1 on focal adhesion molecule expression and type II collagen expression appeared to be dose-dependent (Figs. 9A and 9B). Addition of 5.0 ng/ml IL-1 had a much more dramatic effect on tensin, talin, paxillin, and FAK expression and type II collagen expression than addition of 0.5 ng/ml IL-1.

### Cytochalasin D, an Actin-Disrupting Agent, Inhibits BMP 7-Mediated Increase in Expression of Tensin, Talin, Paxillin, and FAK and Type II Collagen

BMP 7-mediated increase in tensin, talin, paxillin, and FAK expression and type II collagen expression was inhibited by treatment of monolayer-cultured chondrocytes with cytochalasin D (Figs. 10A and 10B). This suggests that the actin cytoskeleton is involved in mediating BMP 7-induced expression of tensin, talin, paxillin, and FAK.

### DISCUSSION

It is well known that articular chondrocyte phenotype is dependent on cell shape/organization of the actin cytoskeleton. In this investigation we have demonstrated that the expression and distribution of focal adhesion proteins, proteins responsible for linking the
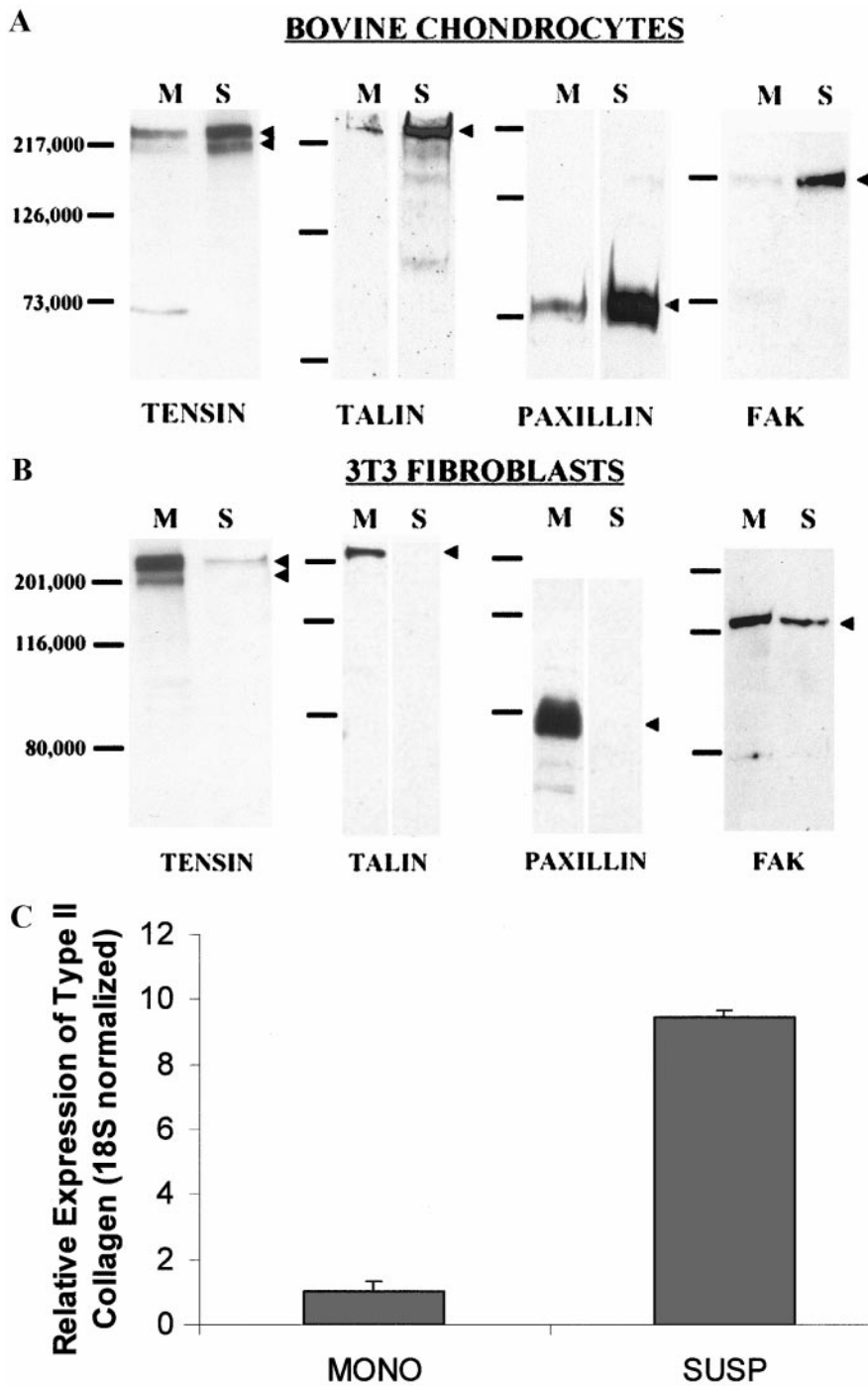
**FIG. 6.** Protein samples extracted from monolayer (M) and suspension (S) cultures of (A) bovine chondrocytes and (B) NIH3T3 fibroblasts were probed with antibodies to tensin, talin, paxillin, and FAK. Interestingly, (A) chondrocytes grown in suspension culture expressed significantly higher levels of tensin, talin, paxillin, and FAK than chondrocytes grown in monolayer culture. The majority of cell types, for example, (B) fibroblasts, express very little or no focal adhesion molecules when grown in suspension culture. Quantitative RT-PCR (TaqMan) data (C) revealed that chondrocytes grown in suspension culture (susp) express higher levels of type II collagen than chondrocytes grown in monolayer culture (mono). Type II collagen is a characteristic marker of the differentiated chondrocyte phenotype.

cytoskeleton to the extracellular matrix, may also influence chondrocyte phenotype. These proteins are tensin, talin, paxillin, and FAK. Our data suggest that external signals, such as BMP 7, IL-1, and cell context, may affect chondrocyte phenotype by altering the expression of these proteins.
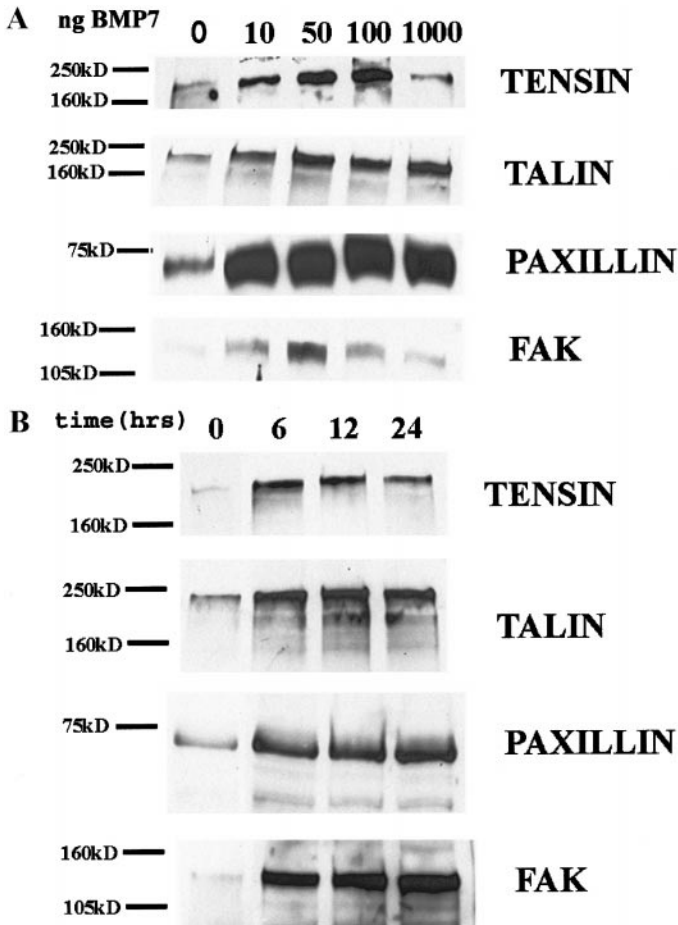
**FIG. 7.** Addition of BMP 7, a morphogen that induces chondrocyte differentiation, to bovine chondrocytes grown in monolayer culture resulted in a significant increase in expression of tensin, talin, paxillin, and FAK. Maximal expression of tensin, talin, paxillin, and FAK was achieved at 50 ng/ml BMP 7 (A) and as early as 6 h after treatment with BMP 7 (B).

sible for the linkage of the actin cytoskeleton to the extracellular matrix we propose that focal adhesion expression and distribution influence organization of the actin cytoskeleton. In suspension culture the actin cytoskeleton forms of a delicate cortical ring of actin that runs parallel to the plasma membrane. The actin cytoskeleton appears to make many contacts with the plasma membrane and this may explain why expression of focal adhesion proteins is much greater in suspension culture. In monolayer culture large struts of actin extend from one edge of the cell to the other and the actin cytoskeleton appears to make far fewer contacts with the plasma membrane. Interestingly, focal adhesion size differs between chondrocytes grown in monolayer and suspension culture. Focal adhesions observed in monolayer culture were much larger than those observed in suspension culture. It is possible that these differences in focal adhesion size relate to the
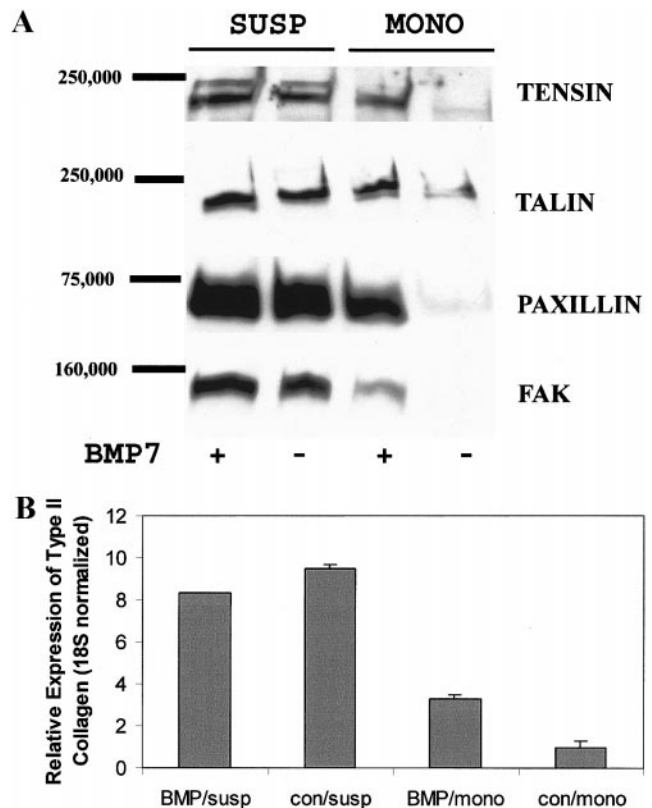


**FIG. 8.** (A) Addition of BMP 7 to bovine chondrocytes grown in suspension culture (susp) did not result in a further increase in expression of tensin, talin, paxillin, or FAK. In contrast, addition of BMP 7 to chondrocytes grown in monolayer culture (mono) resulted in a significant increase in the expression of these molecules. (B) Quantitative RT-PCR (TaqMan) data revealed that addition of BMP 7 to chondrocytes grown in monolayer culture promoted a threefold increase in expression of type II collagen. Addition of BMP 7 to chondrocytes grown in suspension culture did not result in a significant increase in expression of type II collagen.

The expression of focal adhesion proteins by chondrocytes is elevated in suspension compared to monolayer culture. In contrast, NIH 3T3 fibroblasts and most other cell types express focal adhesion proteins maximally in monolayer compared to suspension. It is worthwhile mentioning that unlike most cell types chondrocytes are capable of synthesizing significant amounts of extracellular matrix when cultured in suspension and it is likely to be this characteristic that facilitates the formation of cell–matrix junctions under these conditions. An unexpected finding is that chondrocytes grown in suspension culture express higher levels of focal adhesion junction components than chondrocytes grown in monolayer culture. The distribution of the focal adhesion protein components was also different in chondrocytes grown in suspension and monolayer culture. As focal adhesion junctions are respon-
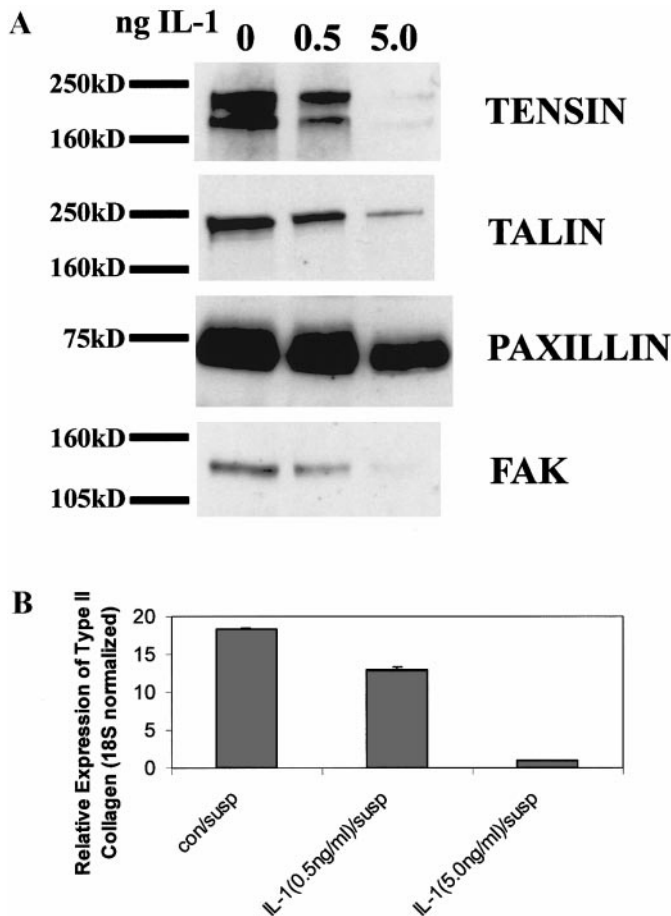
**FIG. 9.** (A) Addition of IL-1, a cytokine that causes chondrocyte dedifferentiation, to bovine chondrocytes grown in suspension culture resulted in a significant, dose-dependent decrease in expression of tensin, talin, paxillin, and FAK. (B) Quantitative RT-PCR (TaqMan) data revealed that the IL-1-dependent decrease in expression of tensin, talin, paxillin, and FAK was concomitant with an IL-1-dependent decrease in type II collagen expression.

chondrocyte phenotype [12, 14–16]. We have demonstrated that culture of chondrocytes in suspension resulted in increased expression of focal adhesion molecules and a concomitant promotion of chondrocyte phenotype. Culture of chondrocytes in monolayer resulted in decreased expression of focal adhesion molecules and a concomitant loss of chondrocyte phenotype. The combined data strongly suggest that expression of focal adhesion proteins and chondrocyte phenotype is linked. It is well known that organization of the actin cytoskeleton can influence chondrocyte phenotype. We have demonstrated that factors which influence chondrocyte phenotype also influence focal adhesion expression, distribution, and size. Linkage of the actin cytoskeleton to the outside of the cell by focal adhesion proteins makes focal adhesion proteins prime candidates for acting as intermediaries between environmental cues and control of cytoskeletal organization and thereby chondrocyte phenotype. It should be noted that it is also possible that increased focal adhesion expression may influence chondrocyte phenotype by activation of signal transduction pathways; tensin, talin, paxillin, and FAK are key players in a number of signal transduction pathways (review: [37]). Interest-
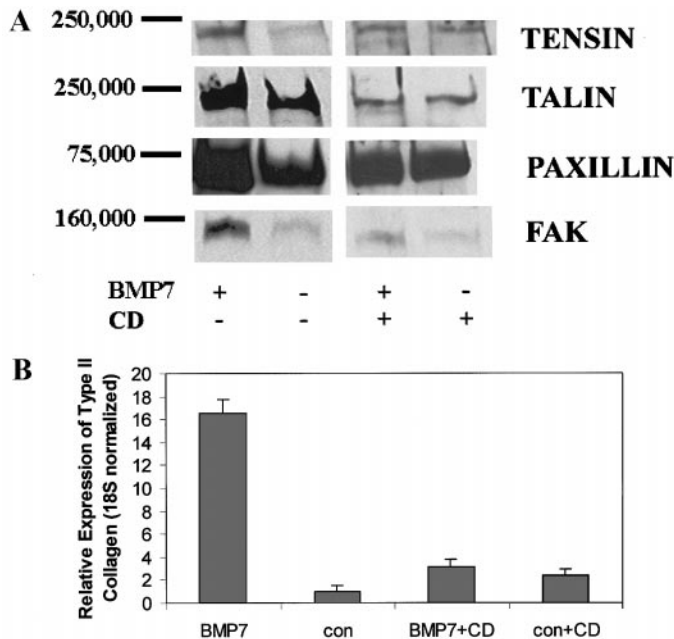


**FIG. 10.** (A) Addition of BMP 7 to monolayer cultures treated with cytochalasin D (CD) did not cause an increase in expression of tensin, talin, paxillin, or FAK (compare the effect of BMP 7 on expression of tensin, talin, paxillin, and FAK in the absence of cytochalasin D (lanes 1 and 2) and in the presence of cytochalasin D (lanes 3 and 4)). Cytochalasin D blocked BMP 7-mediated increase in expression of these molecules. (B) Quantitative RT-PCR (TaqMan) data demonstrated that cytochalasin D also blocked BMP 7-mediated increase in type II collagen expression.

rigidity of the substrate that the cells adhere to. A more rigid substrate, i.e., the culture dish, is likely to promote formation of larger complexes and thereby larger actin struts.

We have demonstrated that factors which influence chondrocyte phenotype also influence expression of focal adhesion proteins. BMP 7 is a morphogen known to induce chondrocyte phenotype [35]. Addition of BMP7 to monolayer cultures resulted in increased expression of focal adhesion proteins and a concomitant promotion of chondrocyte phenotype. IL-1 is a cytokine that is associated with a loss of chondrocyte phenotype [30, 36]. Addition of IL-1 to suspension cultures resulted in decreased expression of focal adhesion proteins and a concomitant loss of chondrocyte phenotype. Last, culture of chondrocytes in suspension promotes chondrocyte phenotype whereas in monolayer there is loss of

ingly, some minor differences were observed in the degree to which expression of tensin, talin, paxillin, and FAK changed in response to the above-mentioned environmental cues. For example, addition of IL-1 to chondrocytes grown in suspension culture resulted in a dramatic reduction in expression of tensin, talin, and FAK, but a relatively smaller decrease in expression of paxillin. It has been observed that focal adhesion composition may differ within a cell [38]. It is possible that focal adhesion composition is dependent on the environmental cues a cell receives and that this is why slight differences in changes in expression levels of tensin, talin, paxillin, and FAK were observed under different conditions.

The inhibition of BMP 7-mediated increase in expression of focal adhesion proteins and concomitant increase in type II collagen expression via disruption of the actin cytoskeleton further confirmed the involvement of the cytoskeleton in control of chondrocyte phenotype. It should be noted that this is the first report to suggest that components of the cytoskeleton may be involved in BMP 7-mediated signaling in chondrocytes. BMPs are known to signal via the Smad pathway and via MAP kinases [26, 27]. Whether focal adhesion proteins interact with these pathways, are part of an independent pathway for BMP signaling, or are downstream of the Smad pathway remains to be determined. Interestingly, other growth factors and cytokines colocalize with focal adhesion proteins and this colocalization is essential for growth factor/cytokine signaling [39, 40].

Chondrocytes maintained in conditions that promote chondrocyte phenotype displayed a distribution of focal adhesion proteins that was very similar to that observed in *ex vivo* tissue sections of articular cartilage. In further support of our hypothesis that focal adhesion expression and distribution influence chondrocyte phenotype, in *ex vivo* tissue sections we noted that the flattened chondrocytes typical of the surface zone expressed fewer focal adhesion proteins than the rounded chondrocytes typical of the deep zone. Chondrocytes present in the surface zone express lower levels of type II collagen than chondrocytes present in the deep zone of articular cartilage. These data suggest that the expression of focal adhesion proteins is important for control of chondrocyte phenotype both *in vivo* and *in vitro.*

In conclusion, we have demonstrated that chondrocyte phenotype is modulated by alterations in expression levels of the focal adhesion components tensin, talin, paxillin, and FAK. We have also demonstrated that BMP 7-mediated promotion of chondrocyte phenotype is dependent on an intact cytoskeleton, suggesting that components of the cytoskeleton are essential for BMP signaling in chondrocytes. The finding that focal adhesion expression modulates chondrocyte phenotype has important implications for cartilage development, and for articular chondrocyte function in health and in diseases such as arthritis.

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