

# CD44 modulates Smad1 activation in the BMP-7 signaling pathway

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**B**one morphogenetic protein 7 (BMP-7) regulates cellular metabolism in embryonic and adult tissues. Signal transduction occurs through the activation of intracellular Smad proteins. In this paper, using a yeast two-hybrid screen, Smad1 was found to interact with the cytoplasmic domain of CD44, a receptor for the extracellular matrix macromolecule hyaluronan. Coimmunoprecipitation experiments confirmed the interaction of Smad1 with full-length CD44—interactions that did not occur when CD44 receptors truncated within the cytoplasmic domain were tested. Chondrocytes overexpressing a trun-

cated CD44 on a background of endogenous full-length CD44 no longer exhibited Smad1 nuclear translocation upon BMP-7 stimulation. Further, pretreatment of chondrocytes with *Streptomyces* hyaluronidase to disrupt extracellular hyaluronan–cell interactions inhibited BMP-7–mediated Smad1 phosphorylation, nuclear translocation of Smad1 or Smad4, and SBE4–luciferase reporter activation. These results support a functional link between the BMP signaling cascade and CD44. Thus, changes in hyaluronan–cell interactions may serve as a means to modulate cellular responsiveness to BMP.

## Introduction

Bone morphogenetic proteins (BMPs) are members of the TGF- $\beta$  superfamily. The inductive capacity of BMPs in tissue development, regeneration, and repair as well as their application in tissue engineering is so widespread that BMPs have been referred to as “body morphogenetic proteins” (Reddi, 1998). The actions of BMPs depend on concentration and on combination with a variety of other factors or ECM components, as well as cell type or cellular age (Hoffman and Gross, 2001). During chondrogenesis, the interactions of cells with other components of the dynamic three-dimensional ECM together with BMP signaling initiate chondrocyte differentiation.

Signaling by BMPs is initiated by their binding to serine/threonine kinase type II and type I receptors and subsequent activation of receptor-activated (R-) Smad proteins (Moustakas et al., 2001). The signaling pathway of BMP-7 (also known as osteogenic protein-1) consists of a principal type

II receptor (ActR-II) that recruits and transphosphorylates the type I receptor ALK2 (Macias-Silva et al., 1998). The ALK2 receptor signals through the R-Smad Smad1. Phosphorylation of Smad1 leads to its dissociation from the type I receptor and the subsequent oligomerization of Smad1 with the mediator Smad4 for nuclear translocation. This signaling pathway is similar to that of BMP-2 and BMP-4 whereby after binding to BMPR-II, the type I receptor ALK3 or ALK6 activate the Smad1 pathway. TGF $\beta$ R-I and TGF $\beta$ R-II transduce TGF- $\beta$  responses through Smad2 and Smad3, two related R-Smads (Moustakas et al., 2001).

Exogenous BMP-7 induces an anabolic response in a variety of connective tissues, including cartilage. BMP-7 stimulates synthesis of aggrecan (the chief proteoglycan of cartilage) and type II collagen (Flechtenmacher et al., 1996) as well as CD44 and hyaluronan synthase-2 (Nishida et al., 2000a,b) in bovine and human articular chondrocytes. Endogenous BMP-7 expression is highest in the cell clusters in osteoarthritic cartilage (Chubinskaya et al., 2000)—areas that may represent attempted repair. Thus, BMP-7 may regulate cartilage development as well as articular cartilage repair and

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Key words: CD44; bone morphogenetic protein; hyaluronan; chondrocyte; Smad1

Abbreviations used in this paper: BMP, bone morphogenetic protein; SBE, Smad-binding element.

homeostasis not only by its stimulation of the synthesis of the major cartilage ECM components, but also with two molecules necessary for the retention of aggrecan, namely hyaluronan and CD44.

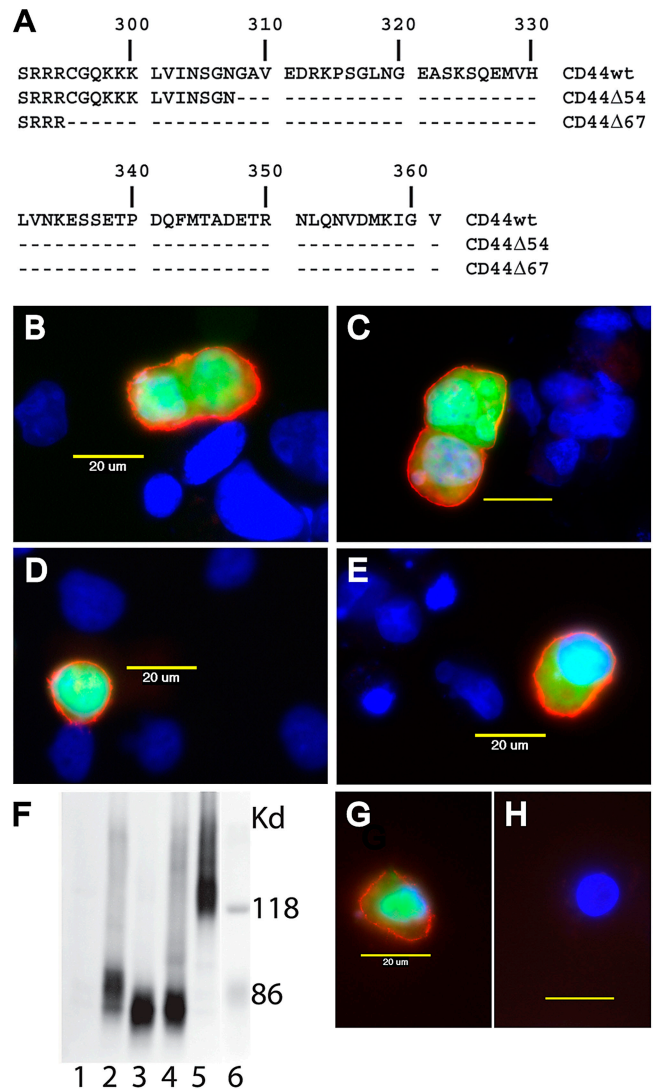
As with other tissues, cell–matrix interactions mediated via transmembrane receptors are responsible for maintaining cartilage homeostasis (Knudson and Knudson, 1993; Knudson, 2003). Articular chondrocytes express integrin as well as nonintegrin ECM receptors (Knudson and Loeser, 2002). These receptors, through interactions with their principal ligands, provide chondrocytes the means to “sense” changes in the ECM environment—changes that may elicit a reparative response, matrix remodeling, or alternatively, cellular quiescence. The CD44 receptor serves as the primary receptor for the ECM macromolecule hyaluronan (Underhill, 1992), mediating both cell–cell and cell–matrix interactions. The CD44H (“hematopoietic”) isoform is a highly glycosylated type I receptor with a 72-aa cytoplasmic domain encoded by exon 20 (Screaton et al., 1992). The mRNA for CD44E (“epithelial”) contains three additional exons (variants 8, 9, and 10) encoding additional protein motifs in the extracellular domain and the same exon 20 encoding the cytoplasmic domain. CD44H is expressed by chondrocytes. In limb development, modulation of hyaluronan–cell interactions during chondrogenic condensation parallels the initiation of CD44 expression (Knudson and Toole, 1987; Rousche and Knudson, 2002). In adult articular chondrocytes, CD44 serves as the critical link to retain hyaluronan–proteoglycan aggregates to the chondrocyte cell surface (Chow et al., 1995). However, new evidence indicates that CD44 participates in the transduction of matrix cues.

In this work, a yeast two-hybrid screen was used to identify proteins that could potentially mediate CD44 signaling. The screen detected binding between the Smad1 protein and a full-length cytoplasmic domain construct of CD44. CD44–Smad1 receptor interactions were confirmed by immunoprecipitations of cell lysates. CD44 receptors truncated in the COOH-terminal cytoplasmic domain (Fig. 1 A) did not have the capacity to interact with Smad1. Chondrocytes overexpressing truncated CD44 in the background of endogenous full-length CD44 no longer exhibited Smad1 nuclear translocation upon BMP-7 stimulation, indicative of a dominant-negative function. Additional evidence is presented to support that hyaluronan–CD44 binding augments the cellular response to BMP-7, including Smad1 phosphorylation and induced nuclear translocation of Smad1 and Smad4. The chondrocyte response to matrix disruption or damage is likely mediated by cross-talk between receptors to ECM components and BMPs (Reddi, 1998). Thus, our data provide further support for the emerging paradigm that cell–matrix interactions modulate the cellular response to morphogens or growth factors.

## Results

### Smad1 and CD44 cytoplasmic domain interaction detected by yeast two-hybrid analysis

Yeast two-hybrid analysis was performed to determine novel proteins interacting with the cytoplasmic domain of CD44. Two CD44 baits were developed, one representing the en-



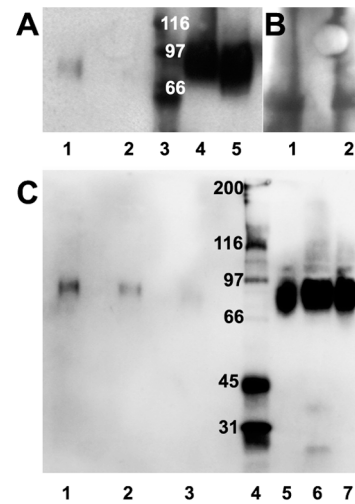
**Figure 1. Human CD44 cytoplasmic domain and CD44 expression by COS-7 and chondrocyte transfectants.** (A) The amino acid sequence of the two yeast two-hybrid baits encoding CD44wt (aa 292–361; full-length CD44 cytoplasmic domain) and CD44Δ54 (aa 292–307; truncation mutant CD44) are depicted. The truncated human CD44 mutants were generated by the introduction of a stop codon at Gly 308 for CD44Δ54 or at Cys 295 for CD44Δ67 in pCD44H. By immunocytochemistry, the CD44 extracellular epitope recognized by the BU-52 mAb was detected at the plasma membrane of GFP-positive COS-7 cells transfected with pCD44H (B), pCD44E (C), and pCD44HΔ67 (E). The V5 epitope was detected after cell permeabilization of COS-7 cells transfected with pCD44H/V5 (D). No positive reaction was observed in the nontransfected COS-7 cells (DAPI nuclear counterstain) in the same fields. After electrophoresis of equivalent protein aliquots from total cell lysates and electroblot transfer, CD44 isoforms expressed by transfected COS-7 cells were visualized with BU-52 (F). Nontransfected COS-7 cells do not express CD44 (lane 1), but after transfection with pCD44H do express a characteristic diffuse band at ~85 kD (lane 2), faster migrating bands after transfection with pCD44HΔ67 (lane 3) or pCD44HΔ54 (lane 4), or a fairly broad band at ~130 kD after transfection with pCD44E (lane 5). Mol wt standards are in lane 6. After transfection with pCD44HΔ67, bovine chondrocytes now express GFP and cell surface human CD44 recognized by the BU-52 mAb (G), whereas no BU-52 immunoreactivity was observed in nontransfected bovine chondrocytes (H). All bars, 20 μm.

tire cytoplasmic domain and another a COOH-terminal truncated version in which the distal 54 amino acids were deleted, CD44 $\Delta$ 54 (Fig. 1 A). Both constructs were expressed to comparable levels in yeast. The yeast two-hybrid analysis revealed an interaction of Smad1 with the full-length cytoplasmic domain of CD44. An interactor matching aa 110–225 of the Smad1 protein was identified upon screening a prey library using the full-length CD44 cytoplasmic tail (aa 292–361) as bait, but not when the shorter CD44 $\Delta$ 54 was tested (aa 292–307). The  $\beta$ -galactosidase score for the CD44–Smad1 interaction hit was recorded at 2.4 SDs above background. No interaction was observed with any other Smad proteins previously characterized in the prey libraries, namely Smads 3, 4, 8, and 9. Subsequently, the full-length cytoplasmic domain bait was tested one-on-one with Smad1 and the original hit was confirmed.

### Expression of CD44 constructs transfected into COS-7 cells and chondrocytes

Two model systems were used in this paper, COS-7 cells that do not express CD44 and bovine articular chondrocytes—cells that express CD44 and exhibit an extensive hyaluronan-dependent matrix. The anti-CD44 mAb BU-52 recognizes an extracellular epitope present in human CD44H and CD44E, as well as the COOH-terminal truncation mutants CD44H $\Delta$ 54 and CD44H $\Delta$ 67 (Fig. 1 A). As shown in Fig. 1 B, nontransfected COS-7 cells (visualized as blue DAPI-stained nuclei) do not exhibit cell surface immunostaining for CD44. Upon transfection with full-length human pCD44H (successfully transfected cells are GFP positive), the COS-7 transfectants now displayed prominent cell surface immunostaining for CD44 with BU-52 (Fig. 1 B, red fluorescence). Transfection of COS-7 cells with other isoforms, pCD44E (Fig. 1 C) and the truncation mutant pCD44H $\Delta$ 67 (Fig. 1 E), followed by BU-52 staining demonstrated that recombinant receptors were successfully processed and transported to the plasma membrane. The pCD44H/V5 construct, containing a COOH-terminal V5-epitope tag, could be detected after cell permeabilization using an anti-V5 mAb (Fig. 1 D). By Western blot analysis of total cell lysates (Fig. 1 F) we confirmed that nontransfected COS-7 cells (lane 1) do not express CD44, as reported in our previous paper (Jiang et al., 2002). Lysates from COS-7 cells transfected with pCD44H exhibited an immunoreactive band at  $\sim$ 85 kD (lane 2), which is the signature molecular mass of full-length, highly glycosylated CD44H. Lysates from COS-7 cells transfected with the larger pCD44E construct exhibited, as expected, a fairly broad band at  $\sim$ 130 kD (lane 5). Lysates from cells transfected with the COOH-terminal truncation mutants pCD44H $\Delta$ 67 (lane 3) or pCD44H $\Delta$ 54 (lane 4) also exhibited protein bands of the expected size range. Thus, in the COS-7 model the expression of CD44 can be selectively and specifically modified.

Primary cultures of bovine chondrocytes represent a more biologically relevant model system. In these cells, transfection of a COOH-terminal truncation mutant pCD44H $\Delta$ 67 is used as a dominant-negative to suppress the activity of the endogenous full-length CD44 (Jiang et al., 2002). Successfully transfected bovine chondrocytes can be visualized readily as GFP-positive cells. In addition, processing and transport of



**Figure 2. Coimmunoprecipitation of CD44 and myc-Smad1.** COS-7 cells cotransfected with pSmad1/myc together with pCD44H, pCD44H $\Delta$ 54, or pCD44H $\Delta$ 67 were lysed, followed by immunoprecipitation using the anti-myc antibody. (A) Immunoprecipitation of Smad1 resulted in the coprecipitation of CD44H (lane 1), but not CD44H $\Delta$ 67 (lane 2) detected with the anti-CD44 mAb BU-52. The supernatants after immunoprecipitation contained nearly equivalent levels of CD44H (lane 4) or CD44H $\Delta$ 67 (lane 5). Mol wt standards are in lane 3. (B) The same blot from A (note that the air bubble watermark in both panels is useful for alignment) was re-probed with an anti-Smad1 antibody to confirm the myc-Smad1 immunoprecipitation. Both lanes 1 and 2 contain Smad1 detected as bands of  $\sim$ 55 kD. (C) COS-7 cells cotransfected with pSmad1/myc together with pCD44H or pCD44H $\Delta$ 54 were treated with BMP-7 for 60 min or left untreated before cell lysis. Immunoprecipitation of myc-Smad1 resulted in the coprecipitation of CD44H (lane 1). A 42% decrease in the band intensity for CD44H was observed (lane 2) when the lysates were prepared from transfectants pretreated with BMP-7. There was no coimmunoprecipitation of Smad 1 and CD44H $\Delta$ 54 (lane 3). The supernatants from these three immunoprecipitations were probed and showed similar high levels of expression of CD44H without (lane 5) or after BMP-7 treatment (lane 6) or expression of CD44H $\Delta$ 54 (lane 7). Mol wt standards are in lane 4.

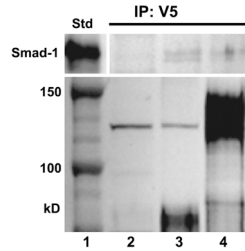
the human CD44H $\Delta$ 67 to the plasma membrane in these cells can be visualized by BU-52 immunostaining (Fig. 1 G, red fluorescence). Bovine chondrocytes express abundant CD44, but are not recognized by anti-human CD44 antibodies such as BU-52 (Fig. 1 H; Aguiar et al., 1999).

### Coimmunoprecipitation of CD44 and Smad1

The interaction between CD44 and Smad1 was confirmed by coimmunoprecipitation assays. COS-7 cells were cotransfected with myc-tagged pSmad1 together with pCD44H or pCD44H $\Delta$ 67. The cells were lysed and proteins were immunoprecipitated using an anti-myc antibody followed by Western blotting with anti-CD44 or anti-Smad mAb. Immunoprecipitation of Smad1 resulted in the coprecipitation of CD44H (Fig. 2 A, lane 1). The COOH-terminal truncation mutant CD44H $\Delta$ 67 was used as a control and displayed no coprecipitation with Smad1 (Fig. 2 A, lane 2). To confirm that CD44H and CD44H $\Delta$ 67 were present in the two cell lysates, the immunoprecipitation supernatants were analyzed. As shown in Fig. 2 A, nearly equivalent levels of CD44H (lane 4) and CD44H $\Delta$ 67 (lane 5) were present in the original cell lysates. To address

**Figure 3. Coimmunoprecipitation of CD44/V5 and endogenous Smad1.**

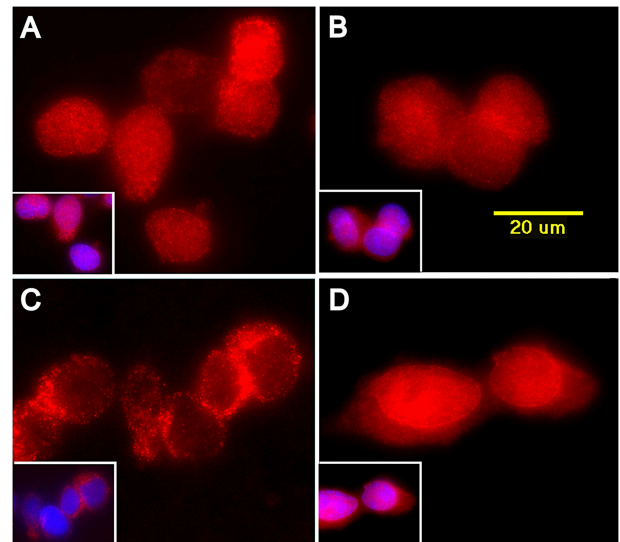
After transfection of COS-7 cells with the control vector (pTracer-EF/V5-His), or with pCD44H/V5 or pCD44E/V5, cell lysates were immunoprecipitated with the anti-V5 mAb, and the immunoblots reacted with an anti-Smad1 (top) or anti-CD44 (bottom) polyclonal H-300 antibody. On the top, endogenous Smad1 at ~55 kD (compared with a Smad1 standard in lane 1) was identified from immunoprecipitates of CD44H (lane 3) and CD44E (lane 4) transfectants, but not from vector control COS-7 cells (lane 2). From the second aliquot of the V5-immunoprecipitate, on the bottom, broad bands were detected at ~85 and at ~130 kD, corresponding to the molecular mass of CD44H (lane 3) and CD44E (lane 4), respectively. Mol wt standards are in lane 1. No CD44 epitope was detected in lysates from COS-7 vector-only controls (lane 2), only the sharp nonspecific band at 124 kD common to all lanes. These CD44–Smad1 coimmunoprecipitations confirm the interaction of CD44H and CD44E with endogenous Smad1.



whether both lysates contained equal concentrations of Smad1, the same immunoprecipitate blot (Fig. 2 A) was re-probed with an anti-Smad1 antibody. As shown in Fig. 2 B, lanes 1 and 2, the anti-myc antibody immunoprecipitated nearly equal levels of myc-tagged Smad1, which can be seen as bands of ~55 kD. Thus, immunoprecipitation of myc-Smad1 resulted in the coprecipitation of CD44H. However, under the same conditions there was no coprecipitation of the CD44 isoform CD44HΔ67 in which most of the cytoplasmic domain has been eliminated.

To determine the effect of BMP-7 stimulation on CD44–Smad1 interactions, COS-7 cells cotransfected with pSmad1/myc together with pCD44H were incubated without or with BMP-7 for 60 min. Again, immunoprecipitation of Smad1/myc with the anti-myc antibody resulted in the coprecipitation of CD44H (Fig. 2 C, lane 1). The amount of CD44H that coprecipitated with Smad1/myc decreased by 42% after pretreatment of the cells with BMP-7 (Fig. 2 C, lane 2). In this same experiment, immunoprecipitation was performed on lysates of COS-7 cells cotransfected with Smad1/myc together with the other COOH-terminal truncation mutant, CD44HΔ54. Corroborating the yeast two-hybrid result, the CD44HΔ54 displayed no coprecipitation with Smad1 (Fig. 2 C, lane 3). Again, to confirm the anti-myc immunoprecipitation the same blot was re-probed using an anti-Smad1 antibody, and all three lanes were found to contain equivalent levels of Smad1 (unpublished data). In addition, analysis of the supernatants from these three immunoprecipitations documented that each lysate contained similar amounts of CD44H without (lane 5) or with BMP-7 pretreatment (lane 6), or CD44HΔ54 (lane 7). Thus, immunoprecipitation of Smad1 resulted in the coprecipitation of CD44H, but not the CD44 cytoplasmic domain truncation mutants (CD44HΔ54 and CD44HΔ67) when all three were expressed to similar extents in COS-7 cells. In addition, the binding of BMP-7 to its receptor appeared to reduce the level of CD44–Smad1 complexes.

To confirm the interaction of CD44 with endogenous Smad1, COS-7 cells were transfected with pCD44 iso-



**Figure 4. Resting CD44<sup>+</sup> COS-7 transfectants show Smad1 cytoplasmic sequestration; BMP-7 treatment initiates Smad1 nuclear translocation.** Parental COS-7 cells, which lack CD44 expression, exhibit diffuse staining of Smad1 protein (red fluorescence) throughout the cytoplasm and the nucleus (A). No change in the diffuse staining pattern of Smad1 protein was observed in parental COS-7 cells after treatment with BMP-7 (B). Stable COS-7 transfectants, expressing cell surface CD44, exhibited Smad1 immunolocalization preferentially to the cytoplasm, and Smad1 staining was nearly absent from the nucleus (C). After treatment of the CD44<sup>+</sup> COS-7 transfectants with BMP-7, the nuclear translocation of Smad1 protein was observed (D). Insets in all panels show two-color fluorescence with the DAPI nuclear stain of the same cells at lower magnification. Bar, 20 μm for the main panels.

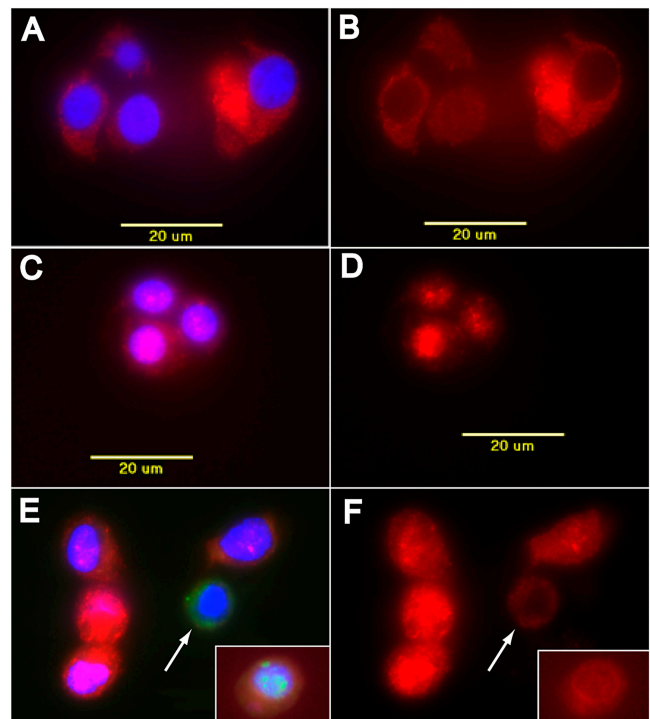
forms containing a V5-epitope tag. COS-7 cells expressing CD44H/V5, CD44E/V5, or empty vector (pTracer-V5) were lysed and immunoprecipitated using an anti-V5 mAb. The immunoprecipitates were split into two aliquots and were resolved by SDS-PAGE followed by electroblotting. In Fig. 3, the top panel depicts the detection of Smad1 with anti-Smad1 antibody and the bottom panel the detection of CD44 with an anti-CD44 pAb. Lane 1 displays recombinant Smad1 as a molecular weight marker (top blot) and as molecular weight markers in the bottom blot. As shown in lane 2, COS-7 cells transfected with empty vector displayed no coprecipitation of endogenous Smad1. However, the cells transfected with either CD44H (lane 3) and the larger CD44E (lane 4) both displayed coprecipitation of endogenous Smad1. The CD44E contains an identical intracellular domain as CD44H, but has a longer extracellular domain sequence. To confirm the immunoprecipitations, the precipitated lysates were probed on a separate blot using an anti-CD44 antibody. From control COS-7 cells (Fig. 3, lane 2, bottom), only a nonspecific band at 124 kD was observed. Lysates from COS-7 cells transfected with CD44H exhibited an ~85-kD band as well as the nonspecific band (lane 3, bottom), and CD44E-transfected cells, a wide band at ~130 kD (lane 4, bottom). These CD44–Smad1 coimmunoprecipitations confirm that endogenous Smad1 present in a mammalian cell type can form interactions with two of the common CD44 isoforms, CD44H and CD44E.

### Nuclear translocation of endogenous Smad1 in COS-7 transfectants expressing CD44

To address whether CD44–Smad1 interactions are important *in situ*, a cell-based, functional approach was investigated. Because nuclear translocation of R-Smad proteins is one of the cellular responses to BMP stimulation, we examined whether changes in the cellular localization of Smad1 were modulated by the expression of CD44. COS-7 cells in suspension were incubated  $\pm$  BMP-7. Parental COS-7 cells, which lack CD44 expression, exhibited diffuse staining of endogenous Smad1 protein throughout the cytoplasm and the nucleus (Fig. 4 A). This is in agreement with a previous report that Smad1 shuttles between these compartments in a resting cell (Xiao et al., 2001). Surprisingly, parental COS-7 cells showed no change in the diffuse staining pattern of Smad1 after incubation with BMP-7 (Fig. 4 B), although nuclear translocation could still be occurring to some extent. This pattern differed considerably from that of a stable CD44<sup>+</sup> transfectant clone of COS-7 cells. Nonstimulated CD44<sup>+</sup> COS-7 cells exhibited endogenous Smad1 immunolocalization predominantly in the cytoplasm with little to no nuclear localization (Fig. 4 C). These results suggest a role for CD44 in Smad1 cytoplasmic sequestration or for presentation to BMP receptors. When the CD44<sup>+</sup> COS-7 transfectants were treated with BMP-7, a clear translocation of endogenous Smad1 protein to the nucleus was observed (Fig. 4 D). The overlaid position of DAPI-stained nuclei are shown in the insets. Thus, upon BMP-7 stimulation of COS-7 transfectants expressing CD44, endogenous Smad1 translocated from the cytoplasm to the nucleus. These data suggest that expression of functional CD44 may be required for the nuclear translocation of Smad1 after BMP-7 stimulation.

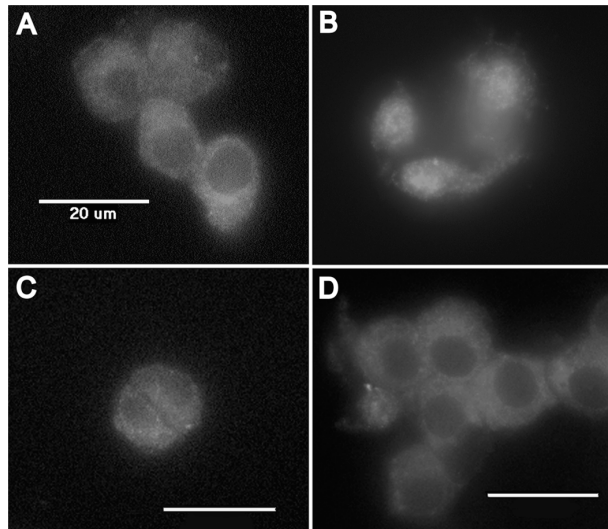
### Inhibition of functional CD44

Next, a loss-of-function approach was used to validate the physiological significance of CD44–Smad1 interactions *in situ*. For these experiments we investigated Smad1 and Smad4 translocation in primary cultures of bovine articular chondrocytes. As shown in Fig. 5 (A and B) control, nonstimulated bovine chondrocytes exhibited cytoplasmic localization of Smad1 (red fluorescence). Upon 60 min of stimulation with BMP-7, nuclear translocation of Smad1 is observed (Fig. 5, C and D). Previous experiments in our laboratories demonstrated that CD44H $\Delta$ 67 transfectants can neither bind nor internalize extracellular hyaluronan (Jiang et al., 2002). Furthermore, overexpression of this isoform in bovine articular chondrocytes results in a dominant-negative effect, inhibiting the biological activity of the endogenous, bovine 85-kD CD44 (Jiang et al., 2002). Bovine chondrocyte cultures were transiently transfected with pCD44H $\Delta$ 67, and transfection was monitored with the co-expression of GFP by chondrocytes within the cultures. In the field depicted in Fig. 5 (E and F), there is one GFP<sup>+</sup> cell (arrow) adjacent to four nontransfected chondrocytes. After treatment with BMP-7 for 60 min, all of the GFP-negative control chondrocytes display Smad-1 nuclear translocation similar to those cells depicted in Fig. 5, C and D. However, the transfected chondrocytes overexpressing dominant-nega-



**Figure 5. Expression of dnCD44 in bovine chondrocytes diminishes Smad1 nuclear translocation.** A, C, and E show the three-color overlay with Smad1 protein in red fluorescence, GFP, and DAPI, whereas the red only is shown in B, D, and F. Control articular chondrocytes exhibited diffuse staining of Smad1 protein in the cytoplasm (A and B). Upon BMP-7 treatment for 60 min, Smad1 translocation to the nucleus was observed (C and D). Transfection of chondrocytes with pCD44H $\Delta$ 67 (E and F, arrow) results in the coexpression of the GFP, but after BMP-7 treatment there was no nuclear staining for Smad1. However, Smad1 translocated to the nucleus of the nontransfected chondrocytes in the same field (E and F) after treatment BMP-7. Chondrocytes transfected with pCD44H $\Delta$ 54 (E and F, inset) also lacked Smad1 nuclear translocation upon BMP-7 treatment. Bars, 20  $\mu$ m.

tive CD44H $\Delta$ 67 (Fig. 5, E and F; GFP<sup>+</sup> cell, arrow) exhibited no nuclear staining for endogenous Smad1 and retained the cytoplasmic localization of Smad1, similar to nonstimulated chondrocytes. As a control, the full-length CD44H construct was overexpressed in bovine chondrocytes, and this did not inhibit Smad1 nuclear translocation in response to BMP-7 (unpublished data). These results were further validated using the other COOH-terminal truncation mutant, CD44H $\Delta$ 54 (see Fig. 1 A). This isoform differs from CD44H $\Delta$ 67 in that it does have the capacity to bind hyaluronan, but is insufficient capacity to interact with Smad1 (as shown in the yeast two-hybrid and immunoprecipitation experiments). As such, overexpression of this construct will not interfere with cell–matrix interactions, but should block intracellular CD44 interactions. As with the transfection of chondrocytes with CD44H $\Delta$ 67, chondrocytes expressing CD44H $\Delta$ 54 did not exhibit nuclear staining for Smad1 after BMP-7 treatment (Fig. 5, E and F, insets). Together, a functional CD44 cytoplasmic domain, distal to the point of the COOH-terminal CD44 $\Delta$ 54 truncation, appears to promote nuclear translocation of Smad1 after BMP-7 stimulation of chondrocytes.

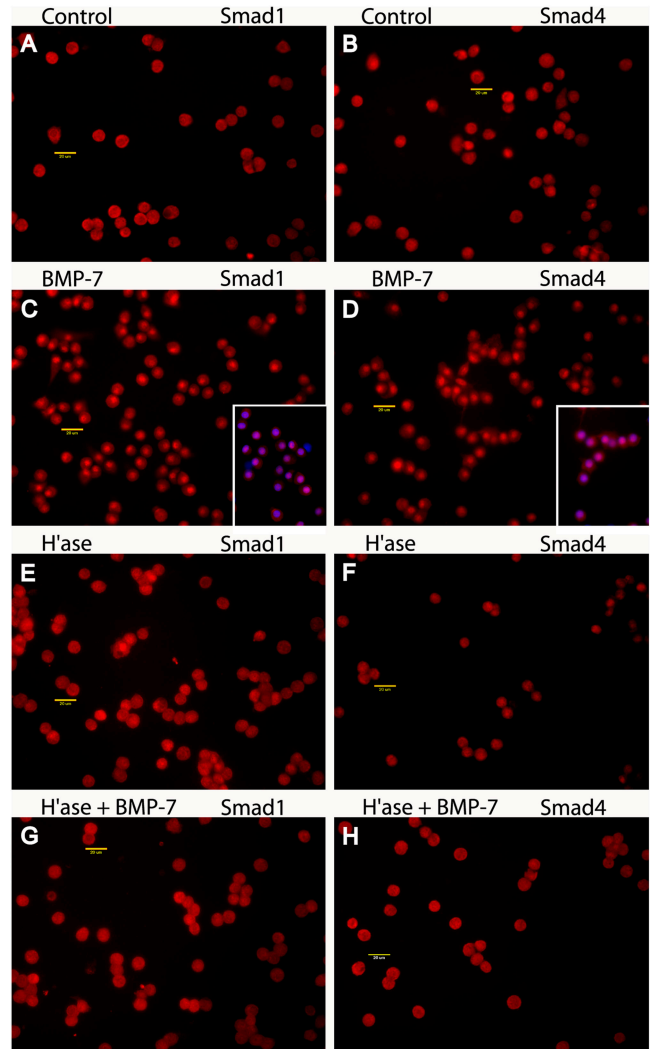


**Figure 6. Nuclear translocation of endogenous Smad1 in bovine chondrocytes.** Smad1 was localized predominantly in the cytoplasm of matrix-intact chondrocytes, with little detectable nuclear immunolocalization (A). After BMP-7 treatment of matrix-intact chondrocytes, Smad1 nuclear immunolocalization is predominant (B). After *Streptomyces* hyaluronidase treatment, immunostaining revealed the maintenance of the diffuse cytosolic location of Smad1 (C). However, BMP-7 treatment of *Streptomyces* hyaluronidase-pretreated chondrocytes did not result in nuclear translocation of Smad1 (D), as seen in the response to BMP-7 by matrix-intact chondrocytes. Bars, 20  $\mu$ m.

### Nuclear translocation of endogenous Smad1 and Smad4 in bovine articular chondrocytes

Next, we explored whether changes in cell–matrix interactions that are mediated through CD44 modulate Smad1-mediated signal transduction. Chondrocytes in culture assemble a prominent pericellular matrix that is anchored by CD44–hyaluronan interactions (Knudson and Knudson, 1993; Knudson, 2003). Treatment with *Streptomyces* hyaluronidase specifically degrades hyaluronan and the pericellular matrix of chondrocytes is removed. Thus, we compared the responsiveness of matrix-intact and matrix-depleted chondrocytes to treatment with BMP-7. BMP-7 responsiveness was first characterized by changes in the cellular distribution of Smad1 and Smad4 after 60 min of BMP-7 stimulation. Control, nonstimulated chondrocytes with an intact pericellular matrix exhibited diffuse immunostaining of Smad1 protein in the cytoplasm (Fig. 6 A). Upon BMP-7 stimulation of chondrocytes for 60 min, endogenous Smad1 translocation to the nucleus was clearly observed as bright nuclear immunostaining (Fig. 6 B). However, in matrix-depleted chondrocytes, subsequent treatment with BMP-7 did not result in Smad1 nuclear translocation (Fig. 6 D). These matrix-depleted chondrocytes continued to exhibit the same nonnuclear, diffuse cytoplasmic localization Smad1 as observed in the nonstimulated chondrocytes (Fig. 6 C).

In Fig. 7, bovine chondrocytes depicted at lower magnification were immunostained for either endogenous Smad1 (A, C, E, and G) or endogenous Smad4 (B, D, F, and H). Control, matrix-intact chondrocytes exhibited diffuse staining for Smad1 (Fig. 7 A) and Smad4 (Fig. 7 B). After BMP-7



**Figure 7. Nuclear translocation of Smad1 and Smad4 in bovine chondrocytes.** Articular chondrocytes were immunostained (red) for either Smad1 (A, C, E, and G) or Smad4 (B, D, F, and H). Control, matrix-intact chondrocytes exhibit diffuse staining for Smad1 (A) and Smad4 (B). After BMP-7 stimulation of chondrocytes for 60 min, Smad1 (C) and Smad4 (D) translocation to the nucleus was observed; insets to these panels show the two-color overlay with DAPI nuclear staining and Smad1 or Smad4 protein. *Streptomyces* hyaluronidase pretreatment alone did not alter the staining patterns observed for Smad1 (E) or Smad4 (F). However, after removal of the pericellular matrix with *Streptomyces* hyaluronidase, subsequent treatment with BMP-7 no longer elicited Smad1 (G) or Smad4 (H) nuclear translocation. Bars, 20  $\mu$ m.

stimulation of matrix-intact chondrocytes, Smad1 translocation to the nucleus (Fig. 7 C) and Smad4 translocation to the nucleus (Fig. 7 D) was observed. *Streptomyces* hyaluronidase pretreatment did not, per se, alter the staining patterns observed for Smad1 (Fig. 7 E) or Smad4 (Fig. 7 F). However, after removal of the pericellular matrix with *Streptomyces* hyaluronidase, subsequent treatment with BMP-7 no longer elicited Smad1 (Fig. 7 G) or Smad4 (Fig. 7 H) nuclear translocation. These results suggest that CD44 receptor occupancy with its ligand, hyaluronan, supports the functional participation of CD44 in the activation of Smad1/4 in the BMP-7 signal transduction pathway.

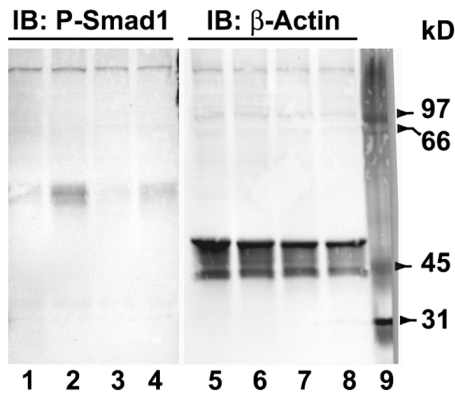


Figure 8. **Western blot analysis of Smad1 phosphorylation.** Total cell lysates were prepared from bovine articular chondrocytes and were analyzed by SDS-PAGE and Western blotting using anti-phospho-Smad1 and anti- $\beta$ -actin antibodies. Lysates from control chondrocytes contained little phospho-Smad1 (lane 1), but lysates from chondrocytes treated for 60 min with BMP-7 showed a strong band for phospho-Smad1 (lane 2). Treatment with *Streptomyces* hyaluronidase alone did not change the intensity of the band for phospho-Smad1 (lane 3) as compared with control (lane 1). However, *Streptomyces* hyaluronidase pretreatment followed by addition of BMP-7 down-regulated phospho-Smad1 content (lane 4). Total  $\beta$ -actin in these four lysates showed little variation in band intensity for the four conditions (lanes 5–8), respectively.

#### Western blot analysis of Smad1 phosphorylation

Smad1 phosphorylation is an initial cellular response to BMP-7 binding to its receptor. Thus, we examined whether disruption of stable CD44–hyaluronan interactions would reduce this response. Total cell lysates were prepared from chondrocytes and were analyzed by SDS-PAGE and Western blotting using anti-Smad1 (to detect total Smad1), anti- $\beta$ -actin, and anti-phospho-Smad1 antibodies (Fig. 8). Lysates from control chondrocytes contained little phospho-Smad1 (lane 1), but lysates from chondrocytes treated for 60 min with 100 ng/ml BMP-7 showed a strong band for phospho-Smad1 (lane 2). Treatment with *Streptomyces* hyaluronidase alone did not change the intensity of the band for phospho-Smad1 (lane 3) in nonstimulated cells as compared with control (lane 1). However, *Streptomyces* hyaluronidase treatment followed by addition of BMP-7 down-regulated phospho-Smad1 content (lane 4). Total Smad1 in the lysates of chondrocytes from these four conditions showed little variation; average band intensity for the four conditions was  $156.25 \pm 7.5$  ODU/mm<sup>2</sup>. Total  $\beta$ -actin in these four lysates also showed little variation (Fig. 8, lanes 5–8); average band intensity for the four conditions was  $68.96 \pm 1.74$  ODU/mm<sup>2</sup>. Normalizing the band intensity for each phospho-Smad1 band with the respective  $\beta$ -actin band intensity was calculated to correct for slight variations in protein concentration. Setting the ratio of phospho-Smad1/ $\beta$ -actin in control lysates to 1.00, the following ratios were calculated for the other culture conditions: +BMP-7 = 23.73; +*Streptomyces* hyaluronidase = 2.8; +*Streptomyces* hyaluronidase +BMP-7 = 10.55. These results suggest that interfering with cellular interactions with the hyaluronan-rich ECM reduces the resultant Smad1 phosphorylation in response to BMP-7 stimulation.

**Smad SBE: AGTATGTCTAGACTGA**

**Smad4: TCGAGAGCCAGACAAAAAGCCAGACATTT  
AGCCAGACAC**

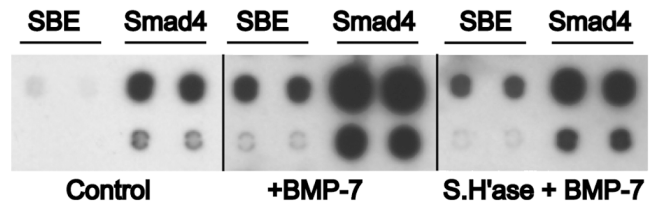


Figure 9. **Protein/DNA array analysis of transcription factor activation after treatment with BMP-7.** Nuclear extracts isolated from bovine articular chondrocytes were analyzed for binding activity to oligonucleotides encoding SBEs (SBE and Smad4; sequences shown at top of figure). Each transcription factor is quantified in duplicate at 1 $\times$  concentration (top two dots) and at 0.1 $\times$  concentration (bottom two dots). Control chondrocytes show resting levels of nuclear Smads. After BMP-7 stimulation for 60 min, chondrocyte nuclear extracts showed increased hybridization to both the Smad SBE and the Smad4 element. Pretreatment with *Streptomyces* hyaluronidase (S.H'ase) before BMP-7 treatment abated the increased hybridization seen with BMP-7 treatment alone.

#### Protein/DNA array analysis of transcription factor activation after treatment with BMP-7

Next, we explored changes in transcription factor activation induced by BMP-7 stimulation of bovine chondrocytes. Using protein/DNA array methodology, nuclear extracts of chondrocytes were analyzed after BMP-7 stimulation without or with pretreatment with *Streptomyces* hyaluronidase. After BMP-7 stimulation for 60 min, many transcription factors in the binding element sequence array remained unchanged (and some undetectable) as compared with control, nonstimulated cells. However, two relevant elements, Smad-binding element (SBE; AGTATGTCTACTACTGA) and the Smad4 element sequence (containing three CAGACA repeats; Jonk et al., 1998), both showed increased hybridization after BMP-7 treatment (Fig. 9). This increased hybridization represents increased DNA-binding activity of these transcription factors. Pretreatment with *Streptomyces* hyaluronidase before BMP-7 treatment abated the increased hybridization seen with BMP-7 treatment alone. Quantification of the dots is shown in Table I. Pax-1 is shown as one example of a transcription factor that showed no change in activity with the treatments, whereas BMP-7 treatment up-

Table I. **Transcription factor activation following treatment of articular chondrocytes with BMP-7**

Sample	Smad4	SBE	Pax-1
Control	111.1 $\pm$ 1.2	2.4 $\pm$ 0.9	53.8 $\pm$ 0.7
+BMP-7	481.2 $\pm$ 4.0	29.2 $\pm$ 0.4	54.4 $\pm$ 1.5
+BMP-7/+S.H'ase	217.7 $\pm$ 11	20.8 $\pm$ 1.4	53.6 $\pm$ 0.9
Control	34.6 $\pm$ 0.1	-	-
S.H'ase	32.0 $\pm$ 2.0	-	-

Nuclei were isolated from control chondrocytes or after treatment with BMP-7, *Streptomyces* hyaluronidase alone (S.H'ase), or co-treatment with BMP-7 and S.H'ase. Transcription factor activation was determined as hybridization signal intensity and presented as the mean  $\pm$  SEM.

regulated Smad activity. Treatment with *Streptomyces* hyaluronidase alone did not change Smad activity in nonstimulated chondrocytes. However, pretreatment with *Streptomyces* hyaluronidase reduced the BMP-7 stimulation of Smad DNA-binding activity. These results confirm Smads as transcription factors differentially activated due to BMP-7 treatment of articular chondrocytes, and that this activation is diminished in matrix-depleted chondrocytes.

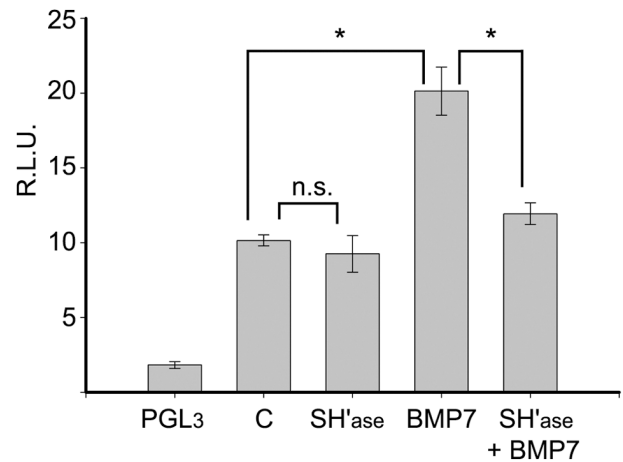
### Analysis of an SBE reporter

Jonk et al. (1998) demonstrated enhanced luciferase activity in response to TGF- $\beta$ , activin, and BMP-7 in HepG2 cells transfected with a PGL3ti-(SBE)<sub>4</sub> plasmid. The PGL3ti-(SBE)<sub>4</sub> contains a concatemeric CAGACA sequence. The PGL3ti-(SBE)<sub>4</sub> plasmid was well expressed after transfection of the C28/I2 chondrocytes, and BMP-7 treatment resulted in a stimulation of luciferase activity above controls (Fig. 10). However, the enhanced luciferase activity was blocked by pretreatment with *Streptomyces* hyaluronidase before the BMP-7 stimulation of chondrocytes (Fig. 10).

### Discussion

In this work, we have uncovered an interaction between CD44 and Smad1. Our data support a functional link between the BMP-7 signal transduction cascade and CD44, and that CD44–hyaluronan interactions promote the cellular response to BMP-7. Thus, modulation or disruption of hyaluronan–cell interactions—during development, tissue remodeling, or degeneration—may decrease the BMP responsiveness of that cell. Most cells express CD44, the principal hyaluronan receptor, and display modified cellular behavior after interaction with hyaluronan (Toole, 2004). Our results suggest that CD44–hyaluronan interactions strengthen the cellular response to BMP-7 that is initiated by Smad1 phosphorylation and nuclear translocation. The disruption of hyaluronan binding to CD44, either by *Streptomyces* hyaluronidase treatment or overexpression of a dominant-negative CD44H $\Delta$ 67, resulted in diminished nuclear translocation of endogenous Smad1 in response to BMP-7. A naturally occurring CD44 isoform with a truncated cytoplasmic domain is generated by alternative splicing of exon 19 in place of exon 20 (Screaton et al., 1992). CD44exon19 is identical to the recombinant CD44H $\Delta$ 67. Interestingly, in a previous paper, we demonstrated that normal human chondrocytes express this CD44 “tail-less” splice variant in proportions varying from 5 to 40% of the total CD44H mRNA (Jiang et al., 2001). When present in the plasma membrane, this variant protein does not bind hyaluronan, would not bind Smad1, and would likely reduce Smad1 nuclear translocation in the BMP signal transduction cascade. Thus, up-regulation of the CD44exon19 splice variant in vivo could be one mechanism to decrease the cellular response to BMPs.

Our observations are consistent with a role for CD44–Smad1 binding in the augmentation of the cellular response to BMPs. CD44 could anchor Smad1 in resting cells for presentation to the type I BMP receptor. After BMP stimulation, we observed a reduction in CD44–Smad1 coimmunoprecipitation as nuclear translocation of Smad1 and Smad4 proceeded. As a precedent, other cytosolic molecules that



**Figure 10. Analysis of an SBE reporter construct in chondrocytes.** The PGL3ti-(SBE)<sub>4</sub> construct was well expressed in untreated control C28I2 chondrocytes (C) compared with the empty PGL3 Basic. BMP-7 treatment resulted in a 2.1-fold stimulation of luciferase activity. However, luciferase activity either decreased or remained the same upon treatment with *Streptomyces* hyaluronidase (S.H'ase) or with *Streptomyces* hyaluronidase pretreatment followed by BMP-7 stimulation of chondrocytes. R.L.U., relative luminescence units; n.s., not significant,  $P > 0.5$ ; \*,  $P < 0.05$  by *t* test.

control the access of R-Smads to the activated type I receptors have been described. Smad anchor for receptor activation (SARA) presents Smad2 or Smad3 to the activated TGF $\beta$  receptor complex. SARA binds to the MH2 domain of Smad2, inducing a conformational change to increase the efficiency of receptor-mediated Smad2 phosphorylation (Wu et al., 2000). The phosphorylation releases Smad2/3 from SARA, allowing Smad4 binding and translocation to the nucleus. As well, the actin-binding protein filamin was found to be a Smad2-binding protein. Filamin-deficient melanoma cells were defective in TGF $\beta$  signaling, but transient expression of filamin restored the response of TGF $\beta$ -reporter gene activation and Smad2 nuclear accumulation (Sasaki et al., 2001). These investigators proposed that filamin might serve as an anchor protein analogous to SARA, to maintain localization of Smad2 near TGF $\beta$  receptors or to present Smad2 in a conformation to facilitate its phosphorylation. CD44 may function in an analogous manner. Gain of CD44 expression in the COS-7 transfectants resulted in both the more unambiguous cytoplasmic localization of Smad1 in the resting transfectants and nuclear translocation of endogenous Smad1 in response to BMP-7 stimulation.

Our current analyses provide additional insight into the mechanism of Smad1 presentation to its cognate receptor kinase as described in other models (Moustakas et al., 2001; Qin et al., 2001). Our data suggest that the Smad1-binding site in the CD44 cytoplasmic domain is distal to the point of the CD44 $\Delta$ 54 truncation. Phosphorylation of serine residues in the cytoplasmic domain of CD44 can modulate receptor function (Lewis et al., 2001). The forkhead associated (FHA) domain of Smad1 is a phosphopeptide-binding domain common to many proteins. Smad1–CD44 binding, potentially via a FHA–phosphoserine interaction, could also participate in the recruitment and/or presentation of Smad1



to its type I receptor. Enzymatic removal of hyaluronan reduced CD44 phosphorylation (unpublished data), which might diminish Smad1-CD44 binding.

Notch and CD44 are receptors without catalytic activity in the cytoplasmic domain, but which undergo regulated intramembrane proteolysis (Cichy and Pure, 2003; Thorne et al., 2004). An extracellular domain cleavage followed by  $\gamma$ -secretase cleavage of the transmembrane domain releases the Notch intracellular domain (ICD; De Strooper et al., 1999) or the CD44 ICD (Lammich et al., 2002; Murakami et al., 2003), which exhibit nuclear translocation. Our results imply the capacity for Smad1 binding to the CD44 ICD. The CD44 ICD can cooperate with the p300/CREB-binding protein (CBP) to activate transcription (Okamoto et al., 2001). Smad1 also acts with p300/CBP to regulate transcriptional activation by BMPs (Pouponnot et al., 1998). In light of these previous reports, our findings suggest that Smad1 together with the CD44 ICD may function as accessory modulators of transcriptional regulation.

The shedding of CD44 after MT1-MMP cleavage of the extracellular domain has been proposed as a mechanism to regulate cell detachment from hyaluronan (Kajita et al., 2001). In addition to proteolytic cleavage of CD44, the receptor is internalized (Aguilar et al., 1999) as part of uptake of hyaluronan to the lysosomal compartment (Knudson and Knudson, 1993). The half-life of cell surface CD44 also decreases in the absence of its ligand, hyaluronan (Aguilar et al., 1999). Together with reduction in CD44 phosphorylation in the absence of ligand or increased expression of CD44 exon 19 as described above, all these are possible mechanisms to limit the participation of CD44 in the BMP signaling pathway.

Our results indicate that the regulation of functional CD44 and CD44 receptor ligation by hyaluronan influences the physiological functions for CD44-Smad1 interaction to modulate the cellular response to BMP-7. Thus, it is possible that changes in CD44-hyaluronan interactions during embryogenesis may regulate the cellular response to BMP. Stable CD44-hyaluronan binding maintains cartilage homeostasis. Cartilage matrix disruption by interleukin-1 (Aydelotte et al., 1992) or fragments of the ECM (Chow et al., 1995; Knudson et al., 2000) results in an imbalance between ECM degradation and anabolic responses by chondrocytes. The intersection of CD44 with the BMP signal transduction pathways may explain this dysregulation. The evidence that Smad1 interacts with the cytoplasmic domain of CD44 supports an emerging paradigm wherein changes in the ECM can modulate BMP signal transduction, culminating in the influence on cellular metabolism.

## Materials and methods

### Cell culture and transfection

Truncated CD44 mutants were generated by the introduction of stop codons (Cys 295: TGT→TGA for  $\Delta 67$ ; or Gly 308: CGA→TGA for  $\Delta 54$ ) in exon 20 of pCD44H (Fig. 1A) using PCR-mediated site-directed mutagenesis as described previously (Jiang et al., 2002). CD44H, CD44H $\Delta 67$ , and CD44H $\Delta 54$  were subcloned into a GFP coexpression vector pTracer-SV40 (Invitrogen; Jiang et al., 2002). Additionally, hCD44H and hCD44E cDNAs were subcloned into the pTracer-EF/V5-His vector (Invitrogen) so the resultant protein would contain a COOH-terminal V5 epitope tag recognized by an anti-V5 mAb (Invitrogen). The parental COS-7 cells express Smad1, but do not express CD44. COS-7 cells were transiently transfected with the various CD44 constructs using LipofectAMINE 2000 (Invitrogen) as described

previously (Jiang et al., 2002). After transfection with pCD44H, a stable clone expressing cell surface CD44 and GFP was isolated using Zeocin selection. After transfection, the anti-human CD44 mAb BU-52 (The Binding Site), which recognizes an extracellular epitope in both CD44H and CD44E, was used to confirm expression. COS-7 cells were also transiently transfected with Smad1 subcloned into pCDNA3.1/myc-His (Invitrogen) or co-transfected with pSmad1/myc and pCD44H, pCD44 $\Delta 67$ , or CD44H $\Delta 54$ .

Bovine articular chondrocytes were isolated (Chow et al., 1995), cultured for a short term in alginate beads (Nishida et al., 2000b), and then released and plated at  $9 \times 10^6$  cells/100-mm dish as monolayer cultures in DMEM + 10% FBS overnight. For transfection, the chondrocytes were pretreated with 5 U/ml *Streptomyces* hyaluronidase (Sigma-Aldrich) in serum-free DMEM. LipofectAMINE 2000 was incubated with pCD44H, pCD44H $\Delta 67$ , or pCD44H $\Delta 54$ , or with the pTracer-SV40 empty vector (facilitator/DNA ratio, 2.5:1), and lipid/DNA complexes were added at 15  $\mu$ g DNA/10<sup>7</sup> cells (Jiang et al., 2002). FBS was added 5 h after transfection to a final concentration of 20%. 24 h after transfection, the medium was replaced with DMEM + 10% FBS and analysis began after 48 h. Transfected bovine chondrocytes express GFP and the human CD44H (or CD44H $\Delta 67$  or CD44H $\Delta 54$ ) protein at the cell surface as detected with BU-52 mAb.

### Yeast two-hybrid screen

Two yeast two-hybrid baits were constructed by subcloning cDNAs encoding aa 292–361 (full-length CD44 cytoplasmic domain) and aa 292–307 (CD44 $\Delta 54$  COOH-terminal truncation) of the human CD44 receptor into the DNA-binding domain fusion vector pMW101 (Watson et al., 1996). The yeast two-hybrid screening protocol was performed as described previously (Slentz-Kesler et al., 2000) using cDNA libraries derived from human prostate and human macrophages.

### Coimmunoprecipitation assays and Western blot analyses

48 h after transfection, the cells were lysed in 20 mM Tris, pH 7.4, with 50 mM NaCl, 5 mM EDTA, 3 mM MgCl<sub>2</sub>, 0.5% Triton-X-100 buffer, with protease (P2714, Sigma-Aldrich) and phosphatase inhibitors (P2850, Sigma-Aldrich). The cell lysates were precleared by protein G-Sepharose (Zymed Laboratories) followed by an overnight incubation at 4°C with anti-myc antibody (Invitrogen) and protein G-Sepharose beads. The beads were washed twice with lysis buffer and the immunoprecipitates were eluted using 100 mM glycine solution, pH 2.6, boiled for 10 min under reducing conditions before loading for SDS-PAGE using a 10% Tris-HCl gel. PVDF membrane was used in the electrotransfer. 5% milk in PBS-Tween was used as a blocking solution. The blot was first probed for CD44 using biotinylated BU-52 antibody (ID Labs), detected with Streptavidin-HRP and ECL reagents (Amersham Biosciences). After 4 d soaking in water, the same blot was next probed for Smad1 after the initial HRP-Streptavidin signal had faded. The anti-Smad-1 pAb (Upstate Biotechnology) was detected using a Vectastain kit (Vector Labs), and incubation with HRP-Streptavidin was followed by ECL reagents. Alternatively, COS-7 transfectants were lysed in 50 mM Tris, pH 7.4, with 150 mM NaCl, 3 mM MgCl<sub>2</sub>, and 0.5% NP-40 buffer, and with protease and phosphatase inhibitors. Pre-cleared lysates were incubated with the anti-V5 mAb and protein G-Sepharose overnight. The immunoprecipitates were analyzed by SDS-PAGE on a 7.5% gel followed by Western blotting. CD44 was detected with an anti-CD44 pAb (H-300; Santa Cruz Biotechnology, Inc.) and endogenous Smad1 was identified using the anti-Smad1 antibody.

Bovine chondrocytes were treated for 60 min with 100 ng/ml BMP-7 (R&D Systems) in the absence or presence of *Streptomyces* hyaluronidase (5 U/ml, 90 min pretreatment followed by a second addition of enzyme at the time of addition of BMP-7), or were left untreated. Total cell lysates were prepared (with the 50 mM Tris buffer above) and analyzed by SDS-PAGE on a 10% gel followed by Western blotting. Total Smad1 and phosphorylated Smad1 were detected with anti-Smad1 and anti-phospho-Smad1 pAbs, respectively (Upstate Biotechnology; Macias-Silva et al., 1998). Actin was detected by a  $\beta$ -actin peptide mAb (AC-15; Sigma-Aldrich). The resultant band intensities were quantified using a Fluor-S imaging system (Bio-Rad Laboratories).

### Immunostaining and Smad nuclear translocation

COS-7 stable transfectants (CD44<sup>+</sup>), control chondrocytes, or chondrocytes after transfection were incubated overnight in media containing only 0.5% FBS. Cells were lifted with nonenzymatic cell dissociation solution (Sigma-Aldrich), incubated for 60 min with 100 ng/ml BMP-7, and then fixed with 2% PFA, permeabilized with 0.2% Triton X-100, and incubated with an anti-Smad1 or an anti-Smad4 antibody (Upstate Biotechnology), which were detected using rhodamine red-X goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Some chondrocytes were pretreated with

*Streptomyces* hyaluronidase. All cells were incubated with DAPI (Molecular Probes, Inc.) as a nuclear counterstain. The cells were viewed using a microscope (Ellipse E600; Nikon) equipped with Y-FI Epi-fluorescence, PLAN-Apochromat 1.4 NA/60× oil and PLAN 0.5 NA/20× objectives. Images were captured digitally in real time using a camera (Spot-RT; Diagnostic Instruments) and were processed using MetaView imaging software (Universal Imaging Corp.).

### Protein/DNA array analysis of transcription factor activation

Nuclear extracts isolated from bovine articular chondrocytes were treated for 60 min with 100 ng/ml BMP-7, in the presence or absence *Streptomyces* hyaluronidase, or untreated control chondrocytes were incubated with biotinylated DNA oligonucleotides of 54 select transcription factor binding element sequences (TransSignal Array; Panomics) (Lam and Li, 2002; Zeng et al., 2003). After isolation of protein/DNA complexes, the samples were denatured and retained binding elements hybridized to membranes containing complementary sequences. Hybridized biotinylated oligonucleotides were visualized using ECL reagents. Each transcription factor was quantified in duplicate at 1× concentration, and in duplicate at 0.1× concentration, per condition.

### Stimulation of Smad-binding element promoter activity in chondrocyte by BMP-7

An SBE-driven luciferase reporter plasmid PGL3ti-(SBE)<sub>4</sub> (Jonk et al., 1998; Althini et al., 2003) was obtained from Dr. Bart Eggen (University of Groningen, Groningen, Netherlands). The immortalized human chondrocyte C-28/12 cells (Tan et al., 2003) were provided by Dr. Mary Goldring (Harvard Institutes of Medicine, Boston, MA). For transfection, the cells were plated 24 h before transfection at a density of 10<sup>5</sup> cells/well in 12-well plates, cultured for 24 h, and transiently cotransfected in serum-free media with 1 μg of the PGL3ti-(SBE)<sub>4</sub> with FuGENE 6 reagent (Roche). After 24 h the media was changed to DMEM and 0.5% FBS. 48 h after transfection, chondrocytes were treated for 24 h with 100 ng/ml BMP-7 in the absence or presence of *Streptomyces* hyaluronidase (5 U/ml, 2-h pretreatment followed by a second addition of enzyme at the time of addition of BMP-7), *Streptomyces* hyaluronidase only for 24 h or no treatment for 24 h. After the treatments the cells were lysed with passive lysis buffer and luciferase activity measured using the dual luciferase assay system (Promega). pSV-β-galactosidase control vector (Promega) activity was measured in all experiments to normalize for transfection efficiency. Graph presented shows mean value ± SEM based on the analysis of triplicate wells from three experiments.

The authors thank Professor Klaus E. Kuettner for many helpful discussions. The Smad1 expression construct was obtained from Dr. Sanjay Kumar (GlaxoSmithKline, Inc., King of Prussia, PA).

This work was supported in part by National Institutes of Health grants P50-AR39239, RO1-AR43384 (W. Knudson), RO1-AR39507 (C.B. Knudson) and grants from the Arthritis Foundation.

Submitted: 25 February 2004

Accepted: 10 August 2004

## References

- Aguiar, D.J., W. Knudson, and C.B. Knudson. 1999. Internalization of the hyaluronan receptor CD44 by chondrocytes. *Exp. Cell Res.* 252:292–302.
- Althini, S., D. Usoskin, A. Kylberg, P. ten Dijke, and T. Ebendal. 2003. Bone morphogenetic protein signalling in NGF-stimulated PC12 cells. *Biochem. Biophys. Res. Commun.* 307:632–639.
- Aydelotte, M.B., R.X. Raiss, B. Caterson, and K.E. Kuettner. 1992. Influence of interleukin-1 on the metabolism of proteoglycan metabolism of cultured bovine articular chondrocytes. *Connect. Tissue Res.* 28:143–159.
- Chow, G., C.B. Knudson, G. Homandberg, and W. Knudson. 1995. Increased CD44 expression in bovine articular chondrocytes by catabolic cellular mediators. *J. Biol. Chem.* 270:27734–27741.
- Chubinskaya, S., C. Merrihew, G. Cs-Szabo, J. Mollenhauer, J. McCartney, D.C. Rueger, and K.E. Kuettner. 2000. Human articular chondrocytes express osteogenic protein-1. *J. Histochem. Cytochem.* 48:239–250.
- Cichy, J., and E. Pure. 2003. The liberation of CD44. *J. Cell Biol.* 161:839–843.
- De Strooper, B., W. Annaert, P. Cupers, P. Saftig, K. Craessaerts, J.S. Mumm, E.H. Schroeter, V. Schrijvers, M.S. Wolfe, W.J. Ray, et al. 1999. A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. *Nature.* 398:518–522.
- Flechtenmacher, J., K. Huch, E.J.-M.A. Thonar, J. Mollenhauer, S.R. Davies, T.M. Schmid, W. Puhl, T.K. Sampath, M.B. Aydelotte, and K.E. Kuettner. 1996. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. *Arthritis Rheum.* 39:478–488.
- Hoffman, A., and G. Gross. 2001. BMP signaling pathways in cartilage and bone formation. *Crit. Rev. Eukaryot. Gene Expr.* 11:23–45.
- Jiang, H., C.B. Knudson, and W. Knudson. 2001. Antisense inhibition of alternatively spliced CD44 variant in human articular chondrocytes promotes hyaluronan internalization. *Arthritis Rheum.* 44:2599–2619.
- Jiang, H., R.S. Peterson, W. Wang, E. Bartnik, C.B. Knudson, and W. Knudson. 2002. A requirement for the CD44 cytoplasmic domain for hyaluronan binding, pericellular matrix assembly and receptor mediated endocytosis in COS-7 cells. *J. Biol. Chem.* 277:10531–10538.
- Jonk, L.J.C., S. Itoh, C.-H. Heldin, P. ten Dijke, and W. Kruijjer. 1998. Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-β, activin, and bone morphogenetic protein-inducible enhancer. *J. Biol. Chem.* 273:21145–21152.
- Kajita, M., Y. Itoh, T. Chiba, H. Mori, A. Okada, H. Kinoh, and M. Seiki. 2001. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J. Cell Biol.* 153:893–904.
- Knudson, C.B. 2003. Hyaluronan and CD44: strategic players for cell-matrix interactions during chondrogenesis and matrix assembly. *Birth Defects Res. Part C. Embryo Today.* 69:174–196.
- Knudson, C.B., and W. Knudson. 1993. Hyaluronan-binding proteins in development, tissue homeostasis and disease. *FASEB J.* 7:1233–1241.
- Knudson, C.B., and B.P. Toole. 1987. Hyaluronate–cell interactions during differentiation of chick embryo limb mesoderm. *Dev. Biol.* 124:82–90.
- Knudson, W., and R.F. Loeser. 2002. CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell. Mol. Life Sci.* 59:36–44.
- Knudson, W., B. Casey, Y. Nishida, W. Eger, K.E. Kuettner, and C.B. Knudson. 2000. Hyaluronan oligosaccharides perturb cartilage matrix homeostasis and induce chondrocytic chondrolysis. *Arthritis Rheum.* 43:1165–1174.
- Lam, R., and X. Li. 2002. An array-based method for specifically profiling multiple transcription factor activity. *Am. Biotechnol. Lab.* 20:22–26.
- Lammich, S., M. Okochi, M. Takeda, C. Kaether, A. Capell, A.K. Zimmer, D. Edbauer, J. Walter, H. Steiner, and C. Haass. 2002. Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Aβ-like peptide. *J. Biol. Chem.* 277:44754–44759.
- Lewis, C.A., P.A. Townsend, and C.M. Isacke. 2001. Ca<sup>2+</sup>/calmodulin-dependent protein kinase mediates the phosphorylation of CD44 required for cell migration on hyaluronan. *Biochem. J.* 357:843–850.
- Macias-Silva, M., P.A. Hoodless, S.J. Tang, M. Buchwald, and J.L. Wrana. 1998. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J. Biol. Chem.* 273:25628–25636.
- Moustakas, A., S. Souchelnytskyi, and C.-H. Heldin. 2001. Smad regulation in TGF-β signal transduction. *J. Cell Sci.* 114:4359–4369.
- Murakami, D., I. Okamoto, O. Nagano, Y. Kawano, T. Tomita, T. Iwatsubo, B. De Strooper, E. Yumoto, and H. Saya. 2003. Presenilin-dependent γ-secretase activity mediates the intramembraneous cleavage of CD44. *Oncogene.* 22:1511–1516.
- Nishida, Y., C.B. Knudson, W. Eger, K.E. Kuettner, and W. Knudson. 2000a. Osteogenic protein-1 stimulates cell-associated matrix assembly by normal human articular chondrocytes. *Arthritis Rheum.* 43:206–214.
- Nishida, Y., C.B. Knudson, K.E. Kuettner, and W. Knudson. 2000b. Osteogenic protein-1 promotes the synthesis and retention of extracellular matrix within bovine articular cartilage and chondrocyte cultures. *Osteoarthritis Cartilage.* 8:127–136.
- Okamoto, I., Y. Kawano, D. Murakami, T. Sasayama, N. Araki, T. Miki, A.J. Wong, and H. Saya. 2001. Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. *J. Cell Biol.* 155:755–762.
- Pouponnot, C., L. Jayaraman, and J. Massague. 1998. Physical and functional interactions of Smads and p300/CBP. *J. Biol. Chem.* 273:22865–22868.
- Qin, B.Y., B.M. Chacko, S.S. Lam, M.P. deCaestecker, J.J. Correia, and K. Lin. 2001. Structural basis of Smad1 activation by receptor kinase phosphorylation. *Mol. Cell.* 8:1303–1312.
- Reddi, A.H. 1998. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat. Biotechnol.* 16:247–252.
- Rousche, K.T., and C.B. Knudson. 2002. Temporal expression of CD44 during embryonic chick limb development and modulation of its expression with

- retinoic acid. *Matrix Biol.* 21:53–62.
- Sasaki, A., Y. Masuda, Y. Ohta, K. Ikeda, and K. Watanabe. 2001. Filamin associates with Smads and regulates transforming growth factor- $\beta$  signaling. *J. Biol. Chem.* 276:17871–17877.
- Screaton, G.R., M.V. Bell, D.G. Jackson, F.B. Cornelis, U. Gerth, and J.I. Bell. 1992. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc. Natl. Acad. Sci. USA.* 89:12160–12164.
- Slentz-Kesler, K., J.T. Moore, M. Lombard, J. Zhang, R. Hollingsworth, and M.P. Weiner. 2000. Identification of the human Mnk2 gene through protein interaction with estrogen receptor beta. *Genomics.* 69:63–71.
- Tan, L., H. Peng, M. Osaki, B.K. Choy, P.E. Auron, L.J. Sandell, and M.B. Goldring. 2003. Egr-1 mediates transcriptional repression of COL2A1 promoter activity by interleukin-1 $\beta$ . *J. Biol. Chem.* 278:17688–17700.
- Thorne, R.F., J.W. Legg, and C.M. Isacke. 2004. The role of the CD44 transmembrane and cytoplasmic domains in co-ordinating adhesive and signalling events. *J. Cell Sci.* 117:373–380.
- Toole, B.P. 2004. Hyaluronan: from extracellular glue to pericellular cue. *Nat. Rev. Cancer.* 4:528–539.
- Underhill, C.B. 1992. CD44: The hyaluronan receptor. *J. Cell Sci.* 103:293–298.
- Watson, M.A., R. Buckholz, and M.P. Weiner. 1996. Vectors encoding alternative antibiotic resistance for use in the yeast two-hybrid system. *Biotechniques.* 21:255–259.
- Wu, G., Y. Chen, B. Ozdamar, C.A. Gyuricza, P.A. Chong, J.L. Wrana, J. Massague, and Y. Shi. 2000. Structural basis of smad2 recognition by the smad anchor for receptor activation. *Science.* 287:92–97.
- Xiao, Z., N. Watson, C. Rodriguez, and H.F. Lodish. 2001. Nucleocytoplasmic shuttling of Smad1 conferred by its nuclear localization and nuclear export signals. *J. Biol. Chem.* 276:39404–39410.
- Zeng, G., L. Gao, T. Xia, T. Tencomnao, and R.K. Yu. 2003. Characterization of the 5'-flanking fragment of the human GM3-synthase gene. *Biochim. Biophys. Acta.* 1625:30–35.