Focal Adhesion Kinase/Src Suppresses Early Chondrogenesis

CENTRAL ROLE OF CCN2*

Received for publication, June 25, 2007, and in revised form, January 22, 2008. Published, JBC Papers in Press, February 13, 2008, DOI 10.1074/jbc.M705175200

Daphne Pala‡1, Mohit Kapoor‡52, Anita Woods‡13, Laura Kennedy‡12, Shangxi Liu‡, Shiqiong Chen‡5, Laura Bursell‡15, Karen M. Lyons‡5, David E. Carter‡5, Frank Beier‡6, and Andrew Leask‡57

From the ‡1Department of Physiology and Pharmacology and ‡5Division of Oral Biology, Canadian Institute of Health Research Group in Skeletal Development and Remodeling, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario N6A 5C1, Canada, ‡6Division of Biological Chemistry and Orthopedic Surgery, David Geffen School of Medicine, University of California, Los Angeles, California 90095, and ‡7London Regional Genomics Centre, Robarts Building, London, Ontario N6A 5C1, Canada

Adhesive signaling plays a key role in cellular differentiation, including in chondrogenesis. Herein, we probe the contribution to early chondrogenesis of two key modulators of adhesion, namely focal adhesion kinase (FAK)/Src and CCN2 (connective tissue growth factor, CTGF). We use the micromass model of chondrogenesis to show that FAK/Src signaling, which mediates cell/matrix attachment, suppresses early chondrogenesis, including the induction of Ccn2, Agc, and Sox6. The FAK/Src inhibitor PP2 elevates Ccn2, Agc, and Sox6 expression in wild-type mesenchymal cells in micromass culture, but not in cells lacking CCN2. Our results suggest a reduction in FAK/Src signaling is a critical feature permitting chondrogenic differentiation and that CCN2 operates downstream of this loss to promote chondrogenesis.

Cartilage is a connective tissue that possesses a wide array of functions, including establishing the skeletal framework during embryogenesis and cushioning joints in adulthood. Chondrocytes, the cell type found in cartilage, generate and maintain the cartilaginous extracellular matrix (ECM), which is crucial for chondrogenic development and homeostasis (1). Formation of the adult skeleton is achieved by intramembranous and endochondral ossification (2, 3). The endochondral skeleton constitutes most skeletal elements of the body and is formed in two main steps. First, cartilage is formed by chondrogenesis. Chondrogenesis is initiated when mesenchymal cells aggregate to form condensations that ultimately determine the shape and location of future bones (2, 3, 4). During this process, a high cell density is achieved that promotes cell-cell interactions resulting in the propagation of signal transduction events necessary for the initiation of chondrogenesis (5). Cells within these condensations begin to express markers typical of early chondrogenic cells. These markers include such proteins as the “Sox trio” of transcription factors (Sox9 and the related factors L-Sox5 and Sox6), type II collagen, and aggrecan (6, 7). Moreover, cells change in morphology from a fibroblast-like appearance to a spheroidal shape (8). Endochondral ossification, a process involving the creation of bone tissue utilizing the cartilage as template, follows (4).

In developing limbs in vivo, cells originating from the lateral plate mesoderm condense and form aggregations (5). In mouse development, formation of these aggregations occurs 10.5–12.5 days post-coitum (dpc) (9). The level and efficiency of overall chondrogenic differentiation, and hence subsequent bone development, is directly correlated to the density of the initial condensation (10). The Sox trio appear to cooperate with each other to co-regulate the expression of chondrogenic markers such as aggrecan and type II collagen. However, much remains unknown about the additional requirements for chondrogenesis. Recently, it was shown that the formation of condensations and cartilage nodules requires adhesive signaling and remodeling of the actin cytoskeleton, via Rho and Rac (11–14). At later stages, adhesive signaling through integrins is involved (15). However, the contribution of cell-ECM interactions in early chondrogenesis is poorly understood. For example, the role of focal adhesion kinase (FAK)/Src, which mediates cell-ECM interactions, in chondrogenesis has not been thoroughly investigated. Moreover, the role of CCN2, a member of the CCN (CYR61, CTGF, NOV) family of pro-adhesive matricellular signaling modulators (16), in early chondrogenesis is unclear.

Culture systems have been developed that promote chondrogenesis. The three-dimensional micromass system is an excellent culture model to promote chondrogenic differentiation of a wide range of mesenchymal cells by providing sufficiently high cell density to promote the cell-cell interactions required for chondrogenesis (10). In this report we use the micromass...
Role of CCN2 in Early Chondrogenesis

cell culture system to probe the role of FAK/Src and CCN2 in early chondrogenesis in vitro.

MATERIALS AND METHODS

Monolayer Cell Culture—Fak<sup>+/+</sup> and Fak<sup>−/−</sup> fibroblast cells (17) (8.5 dpc) were purchased (ATCC). Both cell types lack p53 and have been thereby immortalized. Ccn<sup>2</sup><sup>+/+</sup> and Ccn<sup>2</sup><sup>−/−</sup> mesenchymal cells (13.5 dpc) were isolated and cultured as previously described (18, 19). Monolayer cultures were plated at 2.5 × 10<sup>5</sup> cells/well in a 6-well dish (Nunc). Cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin/amphotericin B (Invitrogen). For Ccn2 rescue experiments, plates were treated overnight with Dulbecco’s modified Eagle’s medium, 0.5% fetal bovine serum, 100 ng/ml CCN2 (EMP; Genetech).

Micromass Cell Culture—Mesenchymal cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin/amphotericin B (Invitrogen). Cells were plated at a density of 1 × 10<sup>5</sup> per 10-μl droplet into each well of a 24-well tissue culture plate (Nunc) and left to adhere for an hour. Once adhered, micromass cultures were given 1 ml of medium. Micromass cultures were grown for a period of 6 days. In experiments involving inhibitor treatment, starting on Day 0, the day of plating, medium was supplemented with the FAK/Src inhibitor PP2 (10 μM) in dimethyl sulfoxide (Me<sub>2</sub>SO) (Calbiochem)), whereas control and untreated samples had to be at least 2-fold, in both sets of experiments. Experiments were performed twice, and -fold changes were identified using the GeneSpring filter. Data presented in Table 1 are an average of these independent studies. The -fold change between treated and untreated samples had to be at least 2-fold, in both sets of experiments, to identify a transcript as being altered.

Western Blot Analysis—Protein was harvested using radioimmunoprecipitation assay lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton-X, 1% deoxycholate, 0.1% SDS, 2 mM EDTA), 50 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>3</sub> (Sigma), supplemented with a protease inhibitor mini complete tablet (Roche Applied Science). Protein concentration was quantified by a kit (BCA; Sigma) as described by the manufacturer’s instructions. Equal amounts of cell lysate (40 μg) and of concentrated medium (20 μl) were subjected to SDS/PAGE and transferred to nitrocellulose (Bio-Rad). Membranes were blocked for 1 h in 5% bovine serum albumin in Tris-buffered saline with 0.01% Tween 20 and incubated with anti-Sox6 (1:200; Sigma), anti-Sox5 (1:1000; Santa Cruz Biotechnology), anti-aggrecan (1:500; R&D Systems), anti-CCN2 antibody (1:2000; Abcam), anti-type II collagen (1:2000; Santa Cruz), or anti-FAK antibody (1:2000; Cell Signaling) overnight at 4 °C. Blots were washed three times for 5 min with Tris-buffered saline with 0.01% Tween 20, followed by the application of appropriate horseradish peroxidase-conjugated secondary antibodies and detection of proteins using ECL™ Western blot detection reagents (Amersham Biosciences) according to the manufacturer’s instructions and visualized using Chemi-Imager™5500 (Alpha Innotech Inc.).

Immunofluorescence—Ccn<sup>2</sup><sup>+/+</sup> and Ccn<sup>2</sup><sup>−/−</sup> cells were plated in monolayer at a density of 12,000 cells/well in a 24-well dish (Falcon) on glass coverslips. Cells were har-
vested and fixed in 4% paraformaldehyde for 30 min at 4 °C. Cells were washed in phosphate-buffered saline (PBS), incubated for 5 min with 0.1% Triton-X in PBS, and rinsed again in PBS. Cells were then incubated in blocking solution containing goat serum (Sigma) in PBS, 1:20, for 30 min at room temperature. Primary antibodies directed to Sox6 were diluted in blocking solution at a concentration of 1:200 and incubated with coverslips for 1 h at room temperature. Coverslips were rinsed in PBS and then incubated with an Alexa-Fluor® 488-conjugated secondary antibody, diluted 1:300 in PBS, for 1 h at room temperature in the dark. Following another wash in PBS, coverslips were mounted in Vectashield anti-fade mounting medium containing 4',6-diamidino-2-phenylindole. Images were taken with a Zeiss Axiophot microscope using Northern Eclipse software (Empix) and exported into Adobe Photoshop.

**Peanut Agglutinin (PNA) Staining**—Micromass cultures were performed as above and were fixed on day 6 of culture in 4% paraformaldehyde at 4 °C for 30 min. Cells were rinsed with PBS and then incubated for 2 h in 50 μg/ml PNA diluted in PBS. Cultures were washed again with PBS, and PNA was detected colorimetrically by diaminobenzidine (Dako Cytomation). Cultures were washed twice with cold PBS, fixed in 100% ethanol for 20 min at −20 °C, and incubated with 0.1% HCl-Alcian blue for 2 h (20). Excess stain was washed off with double distilled water and pictures were taken as described above. Stain was quantified by solubilizing the stain in 6.0 M guanidine hydrochloride for 8 h at room temperature. Absorbance was measured using a spectrophotometer at 620 nm.

**Statistical Analysis**—Data collected from real-time RT-PCR are an average of three trials of samples, from completely independent experiments, run in triplicate. Means were quantified relative to Gapdh, and then data were normalized to day 1 of control per trial or in the case where there is only 1 day being examined, data were normalized to the control sample. Statistical significance was determined by Student’s paired t test or one-way analysis of variance, with a level of significance defined as p < 0.05 using the Bonferroni post-test and GraphPad Prism version 4.00 for Windows.

**RESULTS**

**Loss of FAK Promotes Features of Chondrogenesis, Including Nodule Formation and CCN2 Expression**—The earliest stage of chondrogenesis, namely condensation, involves the migration of mesenchymal cells via cell/ECM interactions and is associated with increased tyrosine phosphorylation of FAK (12). However, the role of FAK in subsequent phases of chondrogenesis, including nodule formation and the induction of chondrogenic genes, is wholly unknown. To specifically assess the effect of loss of FAK on early chondrogenesis, we used the micromass culture system. Micromass cell culture is an established method of inducing chondrogenesis in vitro in a variety of mesenchymal cell types, including embryonic and adult fibroblasts (11, 22–25). To test the contribution of FAK to this process, we subjected Fak+/+ and Fak−/− fibroblasts (Fig. 1a) to high density micromass-induced chondrogenesis. Fak−/− animals die at embryonic day 8.5 (17, 27), precluding use of this type at later embryonic stages. Fibroblasts at 8.5 dpc represent “naive” cells that have not begun to differentiate (9). Fak+/+ and −/− fibroblasts were plated in micromass cultures for 6 days. On day 6 of culture, PNA-stained images (Fig. 1b) were taken of the cultures. To our surprise, loss of FAK expression, even in naive E8.5 fibroblasts, resulted in increased PNA staining, a hallmark of chondrogenic differentiation, in micromass culture as compared with wild-type cells (Fig. 1b). In the Fak+/+ cells, L-Sox5 and Col2a1 mRNA expression was significantly higher than in the wild-type controls (Fig. 1c).

CCN2 is a key modulator of adhesive signaling and promotes chondrogenic gene expression in vivo and in vitro (16, 18). To probe a possible connection between loss of FAK and CCN2 expression, real-time RT-PCR and Western blot analysis of Fak+/+ and −/− fibroblasts in micromass culture for 6 days were used. This analysis revealed that CCN2 protein and mRNA were significantly elevated in the absence of FAK, whereas CCN2 protein and mRNA were not detected in Fak+/+ and −/− fibroblasts (data not shown). Similarly, Sox6 was not induced in Fak−/− E8.5 cells (Fig. 1c). These results surprisingly suggest that the loss of FAK in these naive cells was sufficient to induce some, but not all, features of chondrogenesis.

Loss of Ccn2 Disrupts Expression of Early Chondrogenic Genes in Monolayer Culture—To test the hypothesis that CCN2 acted downstream of the loss of FAK to promote chondrogenesis, it was necessary to employ cells capable of undergoing full chondrogenic differentiation. To investigate this question, we therefore used E13.5 mouse mesenchymal cells, as chondrogenesis occurs in vivo at this stage (9). Microarray analysis of mRNAs isolated from Ccn2+/+ and −/− mesenchymal cells growing in monolayer culture was performed. This analysis revealed that many early chondrogenic genes, such as Agc, Dcn, Hapln (the gene encoding link protein), and Sox6, showed a >2-fold reduction in expression in the absence of CCN2 (Table 1). Moreover, loss of Ccn2 resulted in reduced expression of the CCN family members Ccn1 and Ccn5 (Table 1), both of which have been implicated in bone formation (16). Differences in gene expression between wild-type and knock-out cells revealed by microarray analysis were verified using real-time RT-PCR analysis. As expected, real-time PCR analysis revealed that Ccn2 mRNA expression was observed in wild-type, but not in Ccn2−/− cells (Fig. 2).

Confirming our microarray data, real-time PCR analysis also showed that Ccn2−/− mesenchymal cells possessed a significant decrease in the mRNAs encoded by Ccn1 and Ccn5, Sox6, Agc, Dcn, and the link protein gene Hapln.
Role of CCN2 in Early Chondrogenesis

a) Protein isolated from mouse mesenchymal cells on day 6 was examined for FAK and CCN2 by Western blot analysis. FAK protein expression was absent in Fak−/− mouse embryo fibroblasts. CCN2 expression was up-regulated in the Fak−/− mouse embryo fibroblasts. β-actin was used as a loading control.

b) PNA staining was also performed on day 6. Fak−/− mouse embryo fibroblasts show markedly more condensation formations than Fak+/+ mouse embryo fibroblasts in the PNA-stained micromass cultures, as assessed by diameter of individual micromass cultures. Six different micromass cultures for each condition were examined. Representative cultures and are normalized to control GAPDH levels (each performed in triplicate). *, p < 0.05 using Student’s t test. Note that Agc and Hapln mRNAs were not detected in any samples tested.

c) mRNA expression was significantly increased in Col2a1, RNAs were harvested on day 6, and transcripts were analyzed by real-time RT-PCR. In wild-type mesenchymal cells shows transcripts for L-Sox5 and type II collagen protein expression (Fig. 2). In contrast to these results, we used Western blot analysis to confirm reduced expression of Sox6 and aggrecan protein in the absence of CCN2 (Fig. 3A). Moreover, down-regulation of Sox6 in the Ccn2−/− cells was confirmed by immunofluorescence analyses of Sox6 and aggrecan in reduced glycosaminoglycan production (Fig. 4b). RNAS were harvested on days 1, 3, and 6 of micromass culture, and transcription of chondrogenic genes was analyzed by real-time RT-PCR. In wild-type mesenchymal cells, CCN2 was expressed at constant levels throughout the time course, and, as expected, CCN2 was undetectable in Ccn2−/− cells (Fig. 5). These data suggest that the absence of CCN2 in the micromass culture results in an impaired overall ability of mesenchymal cells to undergo condensation (Fig. 4a). Similarly, Alcian Blue staining revealed that loss of CCN2 expression impaired the overall ability of mesenchymal cells to undergo condensation (Fig. 4a).

TABLE 1

| Gene       | Affy ID     | −Fold Change | Full name                          |
|------------|-------------|--------------|------------------------------------|
| Ctgf, Con2 | 1416953_at  | 29.35        | Connective tissue growth factor (CCN2) |
| Wisp2, Con5| 1419015_at  | 3.73         | Wnt-induced secreted protein-2 (CCN5) |
| Cyr61, Con1| 144234_x_at | 2.12         | Cysteine-rich 61 (CCN1)             |
| Agc        | 1449827_at  | 4.40         | Aggrecan                           |
| Hapln      | 1438020_at  | 2.95         | Link protein                       |
| Dcn        | 1449368_at  | 4.52         | Decorin                            |
| Sox6       | 142767_a_at | 2.81         | Sex-determining region Y (SRY)-box 6 |

FIGURE 1. Loss of FAK in micromass culture increases chondrogenic differentiation. a, for protein analysis, Fak+/+(WT) and −/− (KO) mouse mesenchymal cells (8.5 dpc) were plated in micromass cultures for 6 days. Protein isolated from mouse mesenchymal cells on day 6 was examined for FAK and CCN2 by Western blot analysis. FAK protein expression was absent in Fak−/− mouse embryo fibroblasts. CCN2 expression was up-regulated in the Fak−/− mouse embryo fibroblasts. β-actin was used as a loading control. b, PNA staining was performed on day 6. Fak−/− mouse embryo fibroblasts show markedly more condensation formations than Fak+/+ mouse embryo fibroblasts in the PNA-stained micromass cultures, as assessed by diameter of individual micromass cultures. Six different micromass cultures for each condition were examined. Representative cultures and are normalized to control GAPDH levels (each performed in triplicate). *, p < 0.05 using Student’s t test. Note that Agc and Hapln mRNAs were not detected in any samples tested.
CCN2 alters the balance of collagen and proteoglycan gene expression.

Confirming our prior data using a monolayer culture system, loss of CCN2 resulted in reduced Sox6 expression in micromass culture (Fig. 5). Conversely, Sox9 and L-Sox5 were unaltered in Ccn2−/− micromass cultures on days 1 and 3 and displayed a significant increase in expression in knock-out cells on day 6 (Fig. 5). When regulation of the CCN family was examined, we found that Ccn5 mRNA expression increased significantly during differentiation of wild-type cells but was significantly lower in knock-out cells throughout the culture period (Fig. 5). It is interesting to note that Ccn1 mRNA expression was significantly lower in Ccn2−/− cells on days 1 and 3 of culture but by 6 days of culture similar expression levels of Ccn1 mRNA were observed both in the presence and absence of CCN2 (Fig. 5). Thus, expression of CCN1, a protein possessing functions similar to CCN2 in vitro (16), occurs via a CCN2-dependent fashion in early chondrogenesis but via a CCN2-independent mechanism in later chondrogenesis. Collectively, these data suggest that CCN2 mediates the expression of a subset of chondrogenic genes.

**CCN2 Acts Independently of BMP2**—Members of the transforming growth factor β family, including the BMP proteins that induce chondrogenesis (31), activate CCN2 expression (32, 33). To assess whether CCN2 acted downstream of BMPs to promote chondrogenesis, we subjected Ccn2−/+ and Ccn2−/− mesenchymal cells to micromass-induced chondrogenesis in the presence and absence of added BMP2 (150 ng/ml). We found that BMP2 potentiated chondrogenesis in both Ccn2−/+ and Ccn2−/− mesenchymal cells (Fig. 6), suggesting that BMPs promote chondrogenesis via a CCN2-independent pathway. In fact, the responsiveness of cells to BMP2 was enhanced in the absence of CCN2 (Fig. 6). These results are consistent with previous data using 10T1/2 cells that showed CCN2 blocked BMP action by binding to BMPs, thereby preventing BMPs from interaction with their receptors (34). These results emphasize that, although CCN2 is required for certain features of early chondrogenesis, CCN2-independent pathways play roles during this process.

**Inhibition of FAK/Src Signaling Increases Proteoglycan Gene Expression in a Ccn2-dependent Fashion**—Having shown that FAK suppressed and CCN2 enhanced chondrogenesis, we sought to test the interrelationship between these two processes. To probe the possible connection between FAK, CCN2, and early chondrogenesis, Ccn2+/+ and Ccn2−/− mesenchymal cells were plated in micromass cultures and grown in the presence of Me2SO (as a control) or 10 μM PP2 (a FAK/Src inhibitor) for 6 days. RNAs were harvested and subjected to real-time RT-PCR analysis. Consistent with our observations using E8.5 mesenchymal cells, FAK/Src inhibition induced Ccn2 mRNA (Fig. 7). To assess whether CCN2 operated downstream of the loss of FAK to induce the expression of chondrogenic genes, we assessed whether PP2 altered the mRNA levels of genes previously shown to be CCN2-dependent. We found that Agc, L-Sox6, and Hapln mRNAs were significantly increased in wild-type cells treated with PP2 compared with control cultures treated with Me2SO (as a control) or 10 μM PP2 (a FAK/Src inhibitor) for 6 days. These results confirm that CCN2 is required for the induction of Agc, L-Sox6, and Hapln and show that CCN2 acts downstream of loss of FAK signaling in this process (Fig. 7). Finally, we showed that full-length recombinant CCN2 induced Agc and L-Sox6 expression in Ccn2−/− cells, indicating that these genes are direct targets of CCN2 (Fig. 8). (Expression of Hapln mRNA was undetectable in Ccn2−/− cells with or without CCN2 treatment, indicating that additional proteins work
Role of CCN2 in Early Chondrogenesis

In the absence of CCN2, levels of early chondrogenic matrix-associated proteins Sox6 and aggrecan are decreased. A, protein isolated from Ccn2^+/+ (WT) and ^−/− (KO) mouse mesenchymal cells was cultured in monolayer for 48 h. Conditioned medium and protein extracts were examined for aggrecan and Sox6, respectively, by Western blot analysis. Both Sox6 and aggrecan protein expression were decreased in Ccn2^−/− mouse mesenchymal cells. β-actin was used as a loading control. Densitometry analysis of aggrecan and Sox6, relative to β-actin, shows a significant difference between samples. No difference was seen in the expression of type II collagen and Sox5. Data shown represent means ± S.E. from three trials. *, p < 0.05 using Student’s paired t test.

B, for immunofluorescence analysis, cells were fixed in paraformaldehyde and stained with fluorescein isothiocyanate-labeled (FITC) antibody for Sox6 and with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei. Sox6 was localized around the nuclei in the Ccn2^+/+ mouse mesenchymal cells, whereas expression was markedly decreased in the Ccn2^−/− mouse mesenchymal cells. Six different fields were examined. A representative field is shown.

with CCN2 to induce expression of this gene (not shown). Collectively, these data indicate that a reduction in FAK signaling enhances chondrogenesis in a fashion that is at least partly mediated by CCN2.

**DISCUSSION**

In this report, we investigated the contribution of FAK and CCN2 to early chondrogenesis. Perhaps our most intriguing findings were that FAK suppressed chondrogenic gene expression and that loss of FAK increased CCN2 expression. It is well established that FAK is necessary for focal adhesion turnover and to form connections between the cell and ECM (17, 27). During chondrogenesis, however, cell-cell contacts are favored (35). (Indeed, the micromass culture system is designed to promote chondrogenesis by achieving heightened cell-cell contacts in an environment non-permissive to cell motility.) In our current study, we found that FAK signaling suppressed the expression of chondrogenic markers, including CCN2, aggrecan, and link protein. As an example, the FAK/Src inhibitor PP2 induced expression of CCN2, aggrecan, and link protein in E13.5 mesenchymal cells. Intriguingly, PP2 was unable to induce aggrecan and link protein mRNAs in Ccn2^−/− mesenchymal cells. Overall, our data suggest the novel idea that FAK promotes chondrogenesis by achieving heightened cell-cell contacts in an environment non-permissive to cell motility (16, 36).
Role of CCN2 in Early Chondrogenesis

FIGURE 5. Loss of Ccn2 affects proteoglycan mRNA expression. Ccn2+/+ (WT) and −/− (KO) mouse mesenchymal cells were subjected to micromass cultures for 6 days. For real-time RT-PCR, RNA was harvested on days 1, 3, and 6. Agc, Sox6, and Hapln mRNA expression was significantly reduced in Ccn2−/− mouse mesenchymal cells on all 6 days examined. Conversely, Col2a1, L-Sox5, and Sox9 expression was significantly increased in Ccn2−/− mouse mesenchymal cells. Data shown are relative to GAPDH and represent means ± S.E. from three independent experiments (each performed in triplicate). *p < 0.05 using a two-way analysis of variance. Expression in WT cells on day 1 was taken to represent 1.

FIGURE 6. BMP-2 treatment of Ccn2−/− micromass cultures increases expression of chondrogenic extracellular matrix-associated genes. Ccn2+/+ (WT) and −/− (KO) mouse mesenchymal cells were subjected to micromass culture for 6 days. RNA was harvested on day 3, and transcripts were analyzed by real-time RT-PCR. a–c, induction of Agc, Sox6, and Col2a1 mRNA is greater in Ccn2−/− mouse mesenchymal cells compared with Ccn2+/+ mouse mesenchymal cells. Data shown are relative to GAPDH and represent means ± S.E. from three independent experiments (each performed in triplicate). *p < 0.05 using Student’s paired t test. Fold increase in response to BMP-2 is shown.

cells subjected to micromass culture, induction was not observed of genes shown to be CCN2-dependent using differentiation-competent E13.5 cells. Moreover, recombinant CCN2, which partially rescued the reduced expression of mRNAs encoding Sox6 and aggrecan mRNAs in Ccn2−/− cells, was not able to restore mRNA encoding link protein. Collectively, these observations are consistent with the notion that the mere presence of CCN family members is insufficient to recapitulate the entire range of CCN-modulated activity (29, 30, 39). It is possible therefore that, in E13.5 cells, CCN2 may potentiate the action of other chondrogenic signals. The identity of these putative signals is not known (indeed the mechanisms underlying chondrogenesis are poorly understood) and is beyond the scope of the current study. However, it is interesting to note that we found that CCN2 was not required for cells to respond to the potent chondrogenic protein BMP2. Indeed, we found that Ccn2−/− cells were more responsive to BMP2 than Ccn2+/+ cells. These results are fully consistent with previous observations using 10T1/2 cells that CCN2 blocks BMP action (34).

Our results indicating a role for CCN2 in early chondrogenesis are fully consistent with previous data examining the role of CCN2 in bone formation. CCN2 is expressed in mesenchymal cells during conditions of tissue remodeling and repair, including development and wound healing, and in fibroproliferative disorders, including cancer and fibrosis (30, 40). More specifically, CCN2 is expressed in hypertrophic chondrocytes in the growth plate of cartilage (30, 40). A direct role for CCN2 in cartilage and bone formation in vivo has been recently demonstrated. Mice homozygous for a deletion in the Ccn2 gene have expanded hypertrophic zones in their long bones and possess an underdeveloped rib cage (18). CCN2-deficient mice die soon after birth, presumably due to an inability to breathe properly (18). Moreover, Ccn2−/− mice showed reduced aggrecan and link protein expression (18). These latter observations are completely consistent with our results obtained using cultured mesenchymal cells that CCN2 was required for both aggrecan and link protein expression. Type II collagen expression was not affected by loss of CCN2 in our studies; however, it is interesting to note that CCN2 induces expression of type II and X

Role of CCN2 in Early Chondrogenesis
Role of CCN2 in Early Chondrogenesis

Collagen in chondrosarcoma cells in vitro (28). It should be pointed out that the current in vitro studies of CCN2 function pertaining to bone formation are primarily based on evidence obtained from the chondrosarcoma-derived chondrocytic cell line HCS-2/8 or from mature osteoblasts (28, 41–43). These models are perhaps more relevant in suggesting roles for CCN2 in cancer or later stages of development, respectively, rather than chondrogenesis or osteogenesis per se.

It is perhaps surprising that our current results reveal a role for CCN2 in early chondrogenesis yet the phenotype of the Ccn2−/− mice appear to have defects in later stages of bone development, namely endochondral ossification (18). However, as discussed above, failure of cell to undergo proper condensation is believed to have profound downstream effects on the overall quality of both initial chondrogenesis and later bone formation. Moreover, our current study showed that Ccn1 mRNA expression was down-regulated in monolayer culture and on days 1 and 3 of micromass culture. However, Ccn1 expression was similar in Ccn1−/− and Ccn2−/− cells on day 6 of micromass culture, indicating that CCN2-independent mechanisms controlled Ccn1 expression during early, but not later, stages of chondrogenic differentiation. Previous work has shown similarity in function and expression of CCN1 and CCN2 (29, 44, 45). CCN1 is also known to be expressed in chondrocytes and involved in the synthesis of collagen and other ECM components in vitro (46). Therefore, a potential reason why the skeletal malformations seen in the Ccn2−/− animals are not more severe could be due to a functional compensation by CCN1 (and potentially CCN5) during later stages of chondrogenesis. Testing this notion awaits the generation of mice lacking both CCN1 and CCN2.

Several previous studies have examined the relationship between the Sox trio and their cooperative action during the initiation of chondrogenesis (47–49). It is widely accepted that L-Sox5 and Sox6 are necessary for maximal activity of Sox9 (47–49). Although it is believed that the transcription of chondrocyte-specific genes is coordinately regulated by the Sox trio of transcription factors (48, 50), our current results reveal that CCN2 was required for Sox6 and proteoglycan, but not Sox5, Sox9, and Col2a1, transcription. That is, our results reveal that chondrogenic genes need not necessarily be expressed in parallel. Supporting this notion, L-Sox5 and Sox6, but not Sox9, are down-regulated upon depolymerization of the actin cytoskeleton (13). Furthermore, L-Sox5, Sox9, and Col2a1, but not Sox6 and Agc, expression is induced upon Cdc42 overexpression in ATDC5 cells (14). Our current data therefore support a novel concept that Sox genes may show differential regulation during chondrogenesis. It should be also pointed out that, whereas animals deficient in three or more Sox5 or Sox6 alleles have substantial cartilage defects, mice in which either Sox5 or Sox6 have been deleted have a minimal phenotype (49). Moreover, overexpression of Sox6 in Ccn2−/− cells was insufficient to rescue the gene expression defects in this cell type (not shown). Collectively, these observations suggest that alterations in Sox6 expression are not likely to contribute significantly to the chondrogenic defects observed in the CCN2-deficient cells.

In summary, we have shown for the first time that a loss of FAK/Src activity promotes early chondrogenesis, occurring, at
least in part, by promoting CCN2 expression. In turn, CCN2 is required for the production of proteoglycans. Specifically, our results showed that Sox6 and aggrecan expression are CCN2-dependent whereas type II collagen expression is CCN2-independent. Overall, our data provide new and valuable insights into the complex interplay of signaling cascades during early chondrogenesis.

REFERENCES

1. Karsenty, G., and Wagner, E. F. (2002) *Dev. Cell* 2, 389–406
2. Kronenberg, H. M. (2003) *Nature* 423, 332–336
3. Zeitzer, E., and Olsen, B. R. (2003) *Nature* 423, 343–348
4. Karsenty, G. (2003) *Nature* 423, 316–318
5. Cohn, M. J., and Tickle, C. (1996) *Trends Genet.* 12, 253–257
6. Stanton, L.-A., Underhill, T. M., and Beier, F. (2003) *Dev. Biol.* 263, 165–175
7. Seghatoleslami, M. R., Roman-Blas, J. A., Rainville, A. M., Modaressi, R., Danielson, K. G., and Tuan, R. S. (2003) *J. Cell. Biochem.* 88, 1129–1144
8. Wagner, E. F., and Karsenty, G. (2001) *Curr. Opin. Genet. Dev.* 11, 527–532
9. Karsenty, G. (1999) *Genes Dev.* 13, 3037–3051
10. Ahrens, P., Solursh, M., and Reiter, R. (1977) *Dev. Biol.* 60, 69–82
11. Wang, G., Woods, A., Agoston, H., Ulici, V., Glogauer, M., and Beier, F. (2007) *Dev. Biol.* 306, 612–623
12. Bang, O.-S., Kim, E.-J., Chung, J. G., Lee, S.-R., Park, T. K., and Kang, S.-S. (2000) *Biochem. Biophys. Res. Comm.* 278, 522–529
13. Woods, A., and Beier, F. (2006) *J. Biol. Chem.* 281, 13134–13140
14. Woods, A., Wang, G., Dupuis, H., Shao, Z., and Beier, F. (2007) *J. Biol. Chem.* 282, 23500–23508
15. Assodzi, A., Hunziker, E. B., Brakebusch, C., and Fassler, R. (2003) *Genes Dev.* 17, 2465–2479
16. Leask, A., and Abraham, D. J. (2006) *J. Cell Sci.* 119, 4803–4810
17. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* 377, 539–544
18. Chen, Y., Abraham, D. J., Shi-wen, X., Pearson, J. D., Black, C. M., Lyons, K. M., and Leask, A. (2004) *Mol. Biol. Cell* 15, 5635–5646
19. Ivkovic, S., Yoon, B. S., Popoff, S. N., Safadi, F. F., Libuda, D. E., Stephenson, R. C., Daluiski, A., and Lyons, K. M. (2003) *Development* 130, 2779–2791
20. Leask, A., Underhill, T. M., and Beier, F. (2004) *Biochem. J.* 378, 53–62
21. Woods, A., Wang, G., and Beier, F. (2005) *J. Biol. Chem.* 280, 11626–11634
22. James, C. G., Appleton, C. T., Ulici, V., Underhill, T. M., and Beier, F. (2005) *Mol. Biol. Cell* 16, 5316–5333
23. Carlberg, A. L., Pucci, B., Tallapallli, R., Tuan, R. S., and Hall, D. I. (2001) *Differentiation* 67, 128–138
24. Nicoll, S. B., Barak, O., Csoka, A. B., Bhatnagar, R. S., and Stern, R. (2002) *Biochem. Biophys. Res. Commun.* 292, 819–825
25. Gomes, R. R., Jr., Farach Carson, M. C., and Carson, D. D. (2003) *Connect. Tissue Res.* 44, Suppl. 1, 196–201
26. Kennedy, L., Liu, S., Shi-Wen, X., Chen, Y., Eastwood, M., Carter, D. E., Lyons, K. M., Black, C. M., Abraham, D. J., and Leask, A. (2007) *Exp. Cell Res.* 313, 952–964
27. Furuta, Y. D., Ilic, S., Kanazawa, N., Takeda, T., Yamamoto, S., and Aizawa, S. (1995) *Oncogene* 11, 1989–1995
28. Nakanishi, T., Nishida, T., Shimo, T., Kobayashi, K., Kubo, T., Tamatani, T., Tezuka, K., and Takigawa, M. (2000) *Endocrinology* 141, 264–273
29. Kireeva, M., Latinkic, B., Kolesnikova, T., Chen, C., Yang, G., Ahler, A., and Lau, L. (1997) *Exp. Cell Res.* 233, 63–77
30. Shi-Wen, X., Stanton, L., Kennedy, L., Pala, D., Chen, Y., Howat, S. L., Renzoni, E. A., Carter, D. E., Bou-Gharios, G., Stratton, R., Pearson, J. D., Beier, F., Lyons, K. M., Black, C. M., Abraham, D. J., and Leask, A. (2006) *J. Biol. Chem.* 281, 10715–10726
31. Yoon, B. S., and Lyons, K. M. (2004) *J. Cell. Biochem.* 93, 93–103
32. Holmes, A., Abraham, D. J., Sa, S., Shiwen, X., Black, C. M., and Leask, A. (2001) *J. Biol. Chem.* 276, 10594–10601
33. Luo, Q., Kang, Q., Si, W., Jiang, W., Park, J. K., Peng, Y., Li, X., Liao, H. H., Luo, J., Montag, A. G., Haydon, R. C., and He, T. C. (2004) *J. Biol. Chem.* 279, 55958–55968
34. Abreu, J. G., Ketpura, N. I., Reversade, B., and De Robertis, E. M. (2002) *Nat. Cell Biol.* 4, 599–604
35. DeLise, A. M., Fischer, L., and Tuan, R. S. (2000) *Osteoarthritis Cartilage* 8, 309–334
36. Gilmore, A., and Romer, L. (1996) *Mol. Cell. Biol.* 7, 1209–1224
37. Kawai, J., Akiyama, H., Shirige, C., Ito, H., Konishi, J., and Nakamura, T. (1999) *Eur. J. Cell Biol.* 78, 707–714
38. Briggstock, D. R. (2003) *J. Endocrinol.* 178, 169–175
39. Perbal, B. (2004) *Lancet* 363, 62–64
40. Friedrichsen, S., Heuer, H., Christ, S., Winckler, M., Brauer, D., Bauer, K., and Raivich, G. (2003) *Cell Tissue Res.* 312, 175–188
41. Nakanishi, T., Yamaai, T., Asano, M., Nawachi, K., Suzuki, M., Sugimoto, T., and Takigawa, M. (2001) *Biochem. Biophys. Res. Commun.* 281, 678–681
42. Takigawa, M., Nakanishi, T., Kubota, S., and Nishida, T. (2003) *J. Cell Physiol.* 194, 256–266
43. Takigawa, M. (2003) *Drug News Perspect.* 16, 11–21
44. Chen, C., Chen, N., and Lau, L. (2001) *J. Biol. Chem.* 276, 10443–10452
45. Wong, M., Kireeva, M., Kolesnikov, T., and Lau, L. (1997) *Dev. Biol.* 192, 492–508
46. Akiyama, H., Chaboissier, M. C., Martin, I. F., Schell, A., and de Crombrugghe, B. (2002) *Genes Dev.* 16, 2813–2828
47. Lefebvre, V., Li, P., and de Crombrugghe, B. (1998) *EMBO J.* 17, 5718–5733
48. Lefebvre, V., Behringer, R. R., and de Crombrugghe, B. (2001) *Osteoarthritis Cartilage* 9, 69–75
49. Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J. M., Behringer, R. R., de Crombrugghe, B., and Lefebvre, V. (2001) *Dev. Cell* 1, 277–290
50. Okazaki, K., and Sandell, L. J. (2004) *Clin. Orthop. Relat. Res.* 427, (Suppl.) S123–S128