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RESEARCH ARTICLE

CCN2 (Connective Tissue Growth Factor) is essential for extracellular matrix production and integrin signaling in chondrocytes

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Abstract The matricellular protein CCN2 (*Connective Tissue Growth Factor; CTGF*) is an essential mediator of ECM composition, as revealed through analysis of *Ccn2* deficient mice. These die at birth due to complications arising from impaired endochondral ossification. However, the mechanism(s) by which CCN2 mediates its effects in cartilage are unclear. We investigated these mechanisms using $Ccn2^{-/-}$ chondrocytes. Expression of type II collagen and aggrecan were decreased in $Ccn2^{-/-}$ chondrocytes, confirming a defect in ECM production. $Ccn2^{-/-}$ chondrocytes also exhibited impaired DNA synthesis and reduced adhesion to fibronectin. This latter defect is associated with decreased expression of α5 integrin. Moreover, CCN2 can bind to integrin α5β1 in chondrocytes and can stimulate increased expression of integrin α5. Consistent with an essential role for CCN2 as a ligand for integrins, immunofluorescence and Western blot analysis revealed that levels of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK)1/2 phosphorylation were reduced in $Ccn2^{-/-}$ chondrocytes. These findings argue that CCN2 exerts major effects in chondrocytes through its ability to (1) regulate ECM production and integrin α5 expression, (2) engage integrins and (3) activate integrin-mediated signaling pathways.

Keywords Connective tissue growth factor/CCN2 · Integrin α5 · Extracellular signal-regulated kinase (ERK)1/2 · Focal adhesion kinase (FAK) · Chondrocyte · Fibronectin

Abbreviations

bp	base pairs
E	embryonic day
ECM	extracellular matrix
ERK1/2	extracellular signal-regulated kinase 1/2
FAK	focal adhesion kinase
FN	fibronectin
NP 40	Nonidet P-40
rCCN2	recombinant CCN2 protein
VN	vitronectin
WT	wild type

Introduction

Interactions of cells with the extracellular matrix (ECM) play essential roles in morphogenesis and repair (Ekblom et al. 1986). CCN proteins are matricellular molecules that have important roles in these processes (Lau and Lam 1999; Takigawa 2003; Perbal 2004; Perbal and Takigawa 2005; Rachfal and Brigstock 2005; Kubota and Takigawa 2007).

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CCN2 (Connective tissue growth factor (CTGF)) is a cysteine-rich, ECM-associated, heparin-binding protein, with the characteristic structure of CCN proteins: an N-terminal secretory signal, followed by four modular domains (Brigstock 2003).

CCN2 is a major inducer of ECM production in fibrotic disease (Leask and Abraham 2004), but its role in normal tissues is less well understood. CCN2 is highly expressed in cartilage and endothelial cells in vivo (Nakanishi et al. 2000; Friedrichsen et al. 2003; Ivkovic et al. 2003). Previously, we reported that recombinant CCN2 (rCCN2) promotes the proliferation and differentiation of chondrocytes and osteoblasts (Nakanishi et al. 2000). In order to test the physiological relevance of these activities, we generated *Ccn2*-deficient mice (Ivkovic et al. 2003). These die soon after birth, in part as a result of severe skeletal abnormalities associated with impaired chondrocyte proliferation and ECM expression (Ivkovic et al. 2003). The hypertrophic zones of mutants are enlarged, due to defective remodeling of the cartilage ECM by chondroclasts/osteoclasts, impaired invasion by blood vessels, and subsequent inability to support the formation of an osteoid matrix by osteoblasts (Ivkovic et al. 2003). These and other findings indicate that CCN2 promotes endochondral ossification by enhancing the proliferation and maturation of chondrocytes, and the survival of invading endothelial cells (Nakanishi et al. 2000; Ivkovic et al. 2003; Shimo et al. 1999).

A second member of the CCN family, CCN6, is also essential for chondrocyte function. Homozygosity for loss-of-function mutations in *Ccn6* leads to progressive pseudorheumatoid dysplasia in humans (Hurvitz et al. 1999). This disease is a severe form of childhood-onset arthritis, but nothing is known about the role of CCN6 in normal cartilage. CCN1 may also have essential functions in chondrogenesis. Although *Ccn1*$^{-/-}$ mutants die too early to examine skeletal development, CCN1 stimulates chondrocyte proliferation and ECM accumulation in vitro (Wong et al. 1997; Mo et al. 2002). Given that multiple members of the CCN family have essential functions in chondrogenesis, understanding the mechanisms by which these proteins function is an important issue.

CCN2 functions as a ligand for integrins *in vitro* (Lau and Lam 1999; Babic et al. 1999; Leu et al. 2003; Chen et al. 2004; Gao and Brigstock 2004; Hoshijima et al. 2006; Tong and Brigstock 2006). Integrins play a fundamental role in chondrocyte survival, proliferation and differentiation (Hynes 2002; Loeser 2002). Work from a number of laboratories has documented the presence of $\alpha1\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, and $\alpha v\beta5$ integrins in intact cartilage (Loeser 2002). Of these, $\alpha5\beta1$ is thought to be the most abundant of the $\beta1$-containing integrins in fetal cartilage (Häusler et al. 2002). $\alpha5\beta1$ integrin plays multiple roles in chondrocyte survival and function (Enomoto-Iwamoto et al. 1997; Salter et al. 2001; Pulai et al. 2002; Chen et al. 2005), and cartilage-specific $\beta1$-integrin-deficient mice exhibit profoundly abnormal growth plate structure (Aszodi et al. 2003). A role for $\alpha5\beta1$ integrin in the decision chondrocytes make between endochondral ossification and joint formation has also been suggested (Garciadiego-Cázares et al. 2004). The collagen binding integrins $\alpha1\beta1$ and $\alpha10\beta1$ are also expressed in chondrocytes (Camper et al. 1998; Zemmyo et al. 2003). Integrin $\alpha1^{-/-}$ mice are prone to osteoarthritis (Zemmyo et al. 2003) and exhibit impaired cartilage formation during fracture healing (Ekholm et al. 2002). Mice deficient in $\alpha10$ have only minor defects in cartilage (Bengtsson et al. 2005). Chondrocytes also express integrin $\alpha2$ (Kim et al. 2003; Lahiji et al. 2004), but no skeletal defects have been reported in integrin $\alpha2^{-/-}$ mice (Chen et al. 2002; Holtkötter et al. 2002). In summary, loss of function studies reveals roles for integrins $\alpha1$, $\alpha5$, $\alpha10$, and $\beta1$ in intact cartilage, and $\alpha5\beta1$ is a key regulator of multiple aspects of chondrocyte behavior.

We previously reported that *Ccn2*$^{-/-}$ fibroblasts exhibit impaired adhesive signaling and defective adhesion to fibronectin, and that CCN2 is a ligand for $\alpha5\beta1$ integrin in fibroblasts (Chen et al. 2004). CCN2 also regulates $\alpha5\beta1$ integrin expression in rat fibroblasts (Frazier et al. 1996) and human mesangial cells in vitro (Weston et al. 2003). These studies raise the possibility that CCN2 is an essential ligand for integrins in chondrocytes, and/or regulates integrin expression. We used *Ccn2*$^{-/-}$ chondrocytes to examine these possibilities and their potential relevance to the chondrodysplasia observed in *Ccn2*$^{-/-}$ mice.

Materials and methods

Reagents

Dulbecco's modified Eagle Medium (DMEM) and Minimum Essential (αMEM) Medium Alpha (αMEM) were purchased from Invitrogen (Grand Island, NY); fetal bovine serum (FBS) was from Omega Scientific (Tarzana, CA). Plastic dishes and plates were obtained from Becton-Dickinson (Frabkin Lakes, NJ). Isotopes were from ICN Biomedicals (Aurora, OH) and PerkinElmer, (Boston, MA). Trizol reagent was from Invitrogen; X-ray films were from Kodak (Rochester, NY). Anti-ERK1/2, anti-phospho-ERK1/2, and anti-phoshpho-MEK1/2 were from Cell Signaling Technology (Beverly, MA); anti-FAK, anti-phospho-FAK, anti-integrin $\alpha5\beta1$ for immunoprecipitation (MAB1969), and anti-integrin $\alpha5$ for Western blotting (AB1921) and anti-vimentin were from Chemicon

(Temecula, CA), anti-aggrecan was from Abcam (Cambridge, MA); anti-collagen type II was from Cosmo Bio (Tokyo, Japan); anti-β-actin was from Sigma (St Louis, MO); anti-phospho-tyrosine was from Zymed (San Francisco, CA). Alexa fluor conjugated phalloidin was from Molecular Probes (Eugene, OR). Anti-CTGF/CCN2 serum was as described previously (Nakanishi et al. 2000). Fibronectin (FN) and vitronectin (VN) were from Sigma. rCCN2 was prepared as described (Asano et al. 2005).

Isolation of chondrocytes

Primary cultures of chondrocytes isolated from the ventral half of the rib cage of embryos were prepared as described previously (Lefebvre et al. 1994). Briefly, rib cages and sterna were dissected from E18.5 embryos, rinsed in phosphate-buffered saline (PBS), and incubated at 37°C for 1 h in 2 mg/ml pronase E (Sigma) in PBS, rinsed again with PBS, and then incubated with bacterial collagenase (3 mg/ml: collagenase D; Sigma) in DMEM at 37°C under 7.5% CO_2 for 1 h until soft tissues detached from the cartilage after gentle titration. The cartilage was then further digested with collagenase D for 2 h. Residual bony parts were discarded. The remaining cell suspension was filtered through a 70-μm cell strainer (Becton Dickinson) and rinsed in αMEM containing 10% serum. The isolated chondrocytes were seeded at a density of 1×10^5 cells/cm^2 into 6-well multiplates in αMEM containing 10% FBS, and were then cultured at 37°C under 5% CO_2. Cells derived from each embryo were plated out independently, and tail samples were collected for genotyping.

Adhesion assays

Tissue culture plates (96-well multiplates) were coated with bovine serum albumin (BSA), FN, or VN by incubation overnight at 37°C in 10 μl of PBS containing 5 μg of the protein. The coated plates were then blocked with 1% BSA for 1 h at 37°C, and washed with PBS. For the adhesion assay in Fig. 4c, 96-well plates coated with rCCN2 (5 μg/well) overnight at 37°C. The coated plates were then blocked with a 1:10 dilution of anti-α5 integrin antibody (AB1921, Chemicon) or normal rabbit anti-serum for 1 h at 37°C, and washed with PBS. First passage mouse chondrocytes were inoculated at a density of 5×10^4/well and maintained in αMEM containing 10% FBS. After 60 min, cultures were washed five times with PBS, fixed in 3.5% formaldehyde for 30 min, and stained with 0.5% crystal violet solution (J.T. Baker Chemical, Phillipsburg, NJ) for 30 min. Chondrocyte adhesion was quantified by dye extraction and measurement of absorbance at 550 nm.

Northern blot analysis

Total RNA was prepared using Trizol reagent (Invitrogen). Ten μg of total RNA was subjected to electrophoresis on a 1% formaldehyde-agarose gel and transferred onto Hybond-N+filters (Amersham Pharmacia Biotech). Northern blot analysis was performed as described previously (Nishida et al. 2000, 2002). Oligo-labeled probes for mouse *Ccn2*, and *Collagen type* II *(Col2A1)* were used as described (Nishida et al. 2002); a probe for mouse *aggrecan* was amplified using the following gene-specific primers: (Gen Bank accession no. NM_007424; expected size; 197 bp), forward cggtaccctacagagacacttcaaga and reverse gtgaccctggaacttggt ccaccc.

Real-time reverse transcriptase (RT)-PCR analysis

Quantitative real-time polymerase chain reaction (PCR) was performed by using a LightCycler (Roche; Mannheim, Germany). The primer sequences were as follows: integrin α1 (NM_001033228 expected size 513 bp) forward ttctgatgtcagccctacatt and reverse atagctatggaaaatcgctgaa integrin α2 (NM_008396 expected size 244 bp) forward tgctggctgaaagaccttcacatg and reverse gataa cccctgtcggtacttct; integrin α5 (NM_010577; expected size; 544 bp) forward agcgcatctctcaccatctt and reverse tcaggtt cagtgcgttcttgt; integrin α10 (XM_925721; expected size; 592 bp) forward tggagtctctctccatcc and reverse tcgatgaa cagtcttcctaccagc; integrin β1 (NM_010578; expected size; 451 bp) forward tgttcagtgcagagccttca and reverse cctca tacttcggattgacc; and GAPDH (NM_062046.3; expected size; 171 bp) forward acc-aggtggtctcctctgacttcaa and reverse tactccttggaggccatgt-ggg. forward accaggtggtctc ctctgacttcaa and reverse tactccttggaggccatgtggg. Total RNA was reverse-transcribed to cDNA with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Amplification reactions were performed with a SYBR® Green Real-time PCR Master Mix (Toyobo; Tokyo, Japan).

Western blotting

Chondrocytes were plated on BSA, FN, or VN and maintained in αMEM containing 10% FBS for the indicated times. In some experiments, rCCN2 (50 ng/ml) and/or cycloheximide (20 μg/ml; Sigma) was added to the culture medium. Proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to an Immobilon-P membrane (Millipore) using a tank transfer apparatus (Bio-Rad, Hercules, CA). Cell lysates were prepared and western blot analysis was carried out essentially as described (Kubota et al. 2000).

Evaluation of DNA synthesis and proteoglycan production

Mouse chondrocytes were inoculated at a density of 5×10^4/well into 24-well multiplates, and cultured in αMEM containing 10% FBS for up to 2 days. For determination of cell proliferation, [^{3}H] thymidine incorporation assays were performed on day 0 (2 h after inoculation), 1, and 2. [^{3}H] thymidine (37 MBq/ml, PerkinElmer) was added to the cultures at a final concentration of 370 kBq/ml, and incubation was continued for 4 h. After labeling, cells were harvested and incorporation of radioactivity was determined using liquid scintillation counting. For determination of proteoglycan production, chondrocytes were cultured on chamber slides and stained for alcian blue or toluidine blue as described previously (Atsumi et al. 1990). To quantify the intensity of the staining, the dye was extracted with 1 ml lysis buffer (0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100) overnight at room temperature. The optical density of extracted dye was measured at 600 nm.

Immunoprecipitation analysis

Immunoprecipitation analysis was performed by a modification of a method described previously (Nishida et al. 2004). Briefly, cell layers were solubilized in lysis buffer and a mixture of protease inhibitors. Lysates were immunoprecipitated with antibody or heparin sepharose overnight at 4°C. Antibody complexes samples were collected by incubation with protein G-sepharose (Roche, Mannheim, Germany). Pellets were washed three times with ice-cold PBS, and precipitated proteins were solubilized in reducing SDS sample buffer. Samples were electrophoresed on SDS-polyacrylamide gels and subjected to Western blot analysis.

Indirect immunofluorescence analysis

Cells were inoculated on chamber slides (Nunc Inc., Naperville, IL) coated with FN. After 4 h, cultures were washed with PBS, fixed with 3.5% formaldehyde for 30 min at room temperature, and permeabilized with 0.1% NP-40 in PBS. Indirect immunofluorescence analysis was performed as described previously (Aszodi et al. 2003). Images were obtained using either a confocal laser scanning microscope (Bio-Rad) or a light microscope (Zeiss).

Immunohistochemistry

Mouse femurs were dissected and fixed in 10% formalin overnight at 4°C before being embedded in paraffin. Five micrometer longitudinal sections were mounted on glass slides, deparaffinized and treated with hyaluronidase (25 mg/ml) for 30 min at room temperature. Immunohistochemistry was performed with a Histofine kit (Nichirei; Tokyo, Japan). Color was developed with diaminobenzine, and sections were counterstained with methyl green. Control specimens incubated with diluted non-immune antibody showed no detectable signals.

Statistical analysis

Unless otherwise specified, all experiments were repeated at least twice, and similar results were obtained. Statistical analysis was performed by Student's *t* test. Densitometry was performed using NIH ImageJ (Version 1.38 m).

Results

Characterization of chondrocytes derived from CCN2 mutant mice

Chondrocytes were isolated from rib cages of E18.5 wild type (WT), *Ccn2*$^{+/-}$, and *Ccn2*$^{-/-}$ mice. Cells isolated from mutant and WT mice displayed a typical chondrocyte morphology, and no differences were observed in *Ccn2*$^{-/-}$ chondrocytes compared to WT or heterozygous cells (Fig. 1a). It has been reported that recombinant CCN2 protein promotes chondrocyte proliferation and differentiation (Nakanishi et al. 2000). Therefore, we investigated whether proliferation and/or differentiation were impaired in *Ccn2*$^{-/-}$ chondrocytes. A significant decrease in [^{3}H] thymidine incorporation was observed in *Ccn2*$^{-/-}$ chondrocyte cultures compared to WT cells (Fig. 1b). These data demonstrate that CCN2 is required for normal rates of chondrocyte proliferation. The effect of loss of *Ccn2* on differentiation was examined by staining with toluidine blue and alcian blue to visualize cartilage matrix proteoglycans. As shown in Fig. 1c, staining with both dyes was decreased in *Ccn2*$^{-/-}$ chondrocytes; absorbance at 600 nm of extracted alcian blue shows that *Ccn2*$^{-/-}$ chondrocytes exhibit an approximately 50% decrease in proteoglycan content compared to WT cells. Therefore, ECM production is significantly impaired in *Ccn2*$^{-/-}$ chondrocytes.

To further investigate defects in ECM production in *Ccn2*$^{-/-}$ chondrocytes, we examined the levels of expression of specific markers. *Ccn2*$^{-/-}$ mRNA (Fig. 2a) and protein (Fig. 2b) were undetectable in *Ccn2*$^{-/-}$ chondrocytes, confirming that the previously described targeted *Ccn2* mutation encodes a null allele (Ivkovic et al. 2003). Expression of both type II collagen and aggrecan mRNA and protein were decreased in *Ccn2*$^{-/-}$ chondrocytes, compared with WT cells (Fig. 2a,b; Fig. S1a, Supplementary data). Densitometric analysis confirmed that type II collagen and aggrecan protein levels were substantially

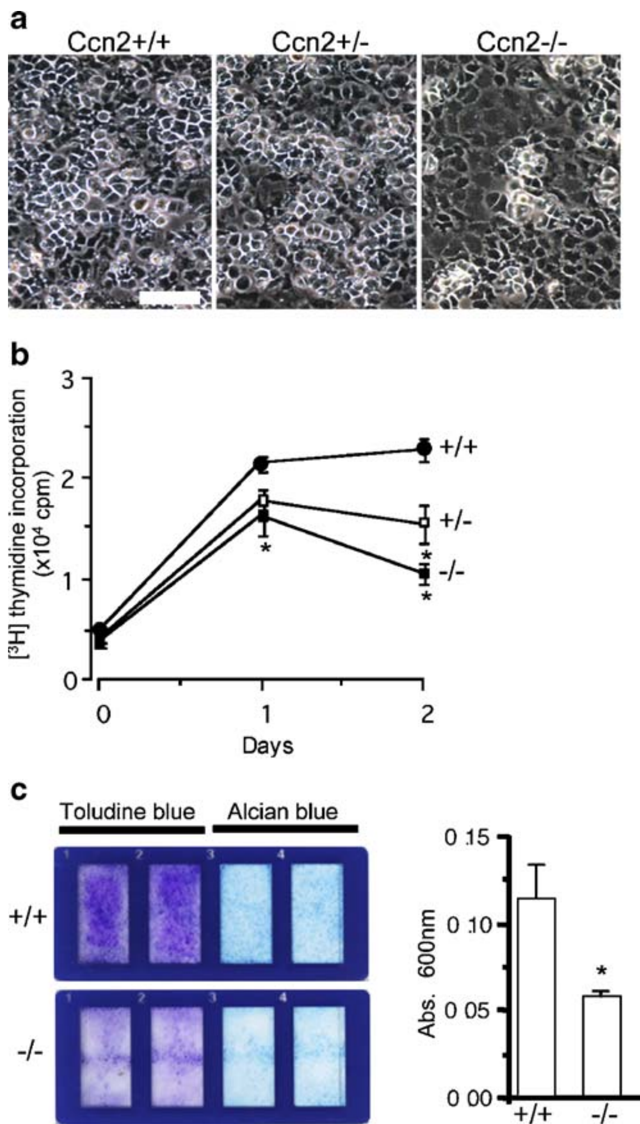

Fig. 1 Morphology and expression of cartilage markers in *Ccn2*$^{-/-}$ chondrocytes. **a** Photomicrographs of mouse chondrocytes. Chondrocytes were grown after one passage until confluent. Bar 200 μm. **b** *Ccn2*$^{+/+}$ (*filled circle*), *Ccn2*$^{+/-}$ (*open square*), and *Ccn2*$^{-/-}$ (*filled square*) chondrocytes were inoculated at a density of 5×10^4/well in 24-well plates, and cultured in αMEM containing 10% FBS. [^{3}H] thymidine incorporation assays were performed on days 0 (2 h after plating), 1, and 2. Each point shows the mean and S.D. for four wells. *Asterisks* indicate significant differences between WT and *Ccn2*$^{-/-}$ cells at the significance level of $p<0.01$. **c** WT and *Ccn2*$^{-/-}$ chondrocytes were cultured until confluent. Duplicate cultures of the cells were fixed in 3.5% formaldehyde, and stained with 0.05% toluidine blue or 1% alcian blue. For quantification of alcian blue staining (*right panel*), stained chondrocytes were dissolved in lysis buffer, and the absorbance was measured at 600 nm. The asterisk indicates a significant difference between WT and *Ccn2*$^{-/-}$ chondro-

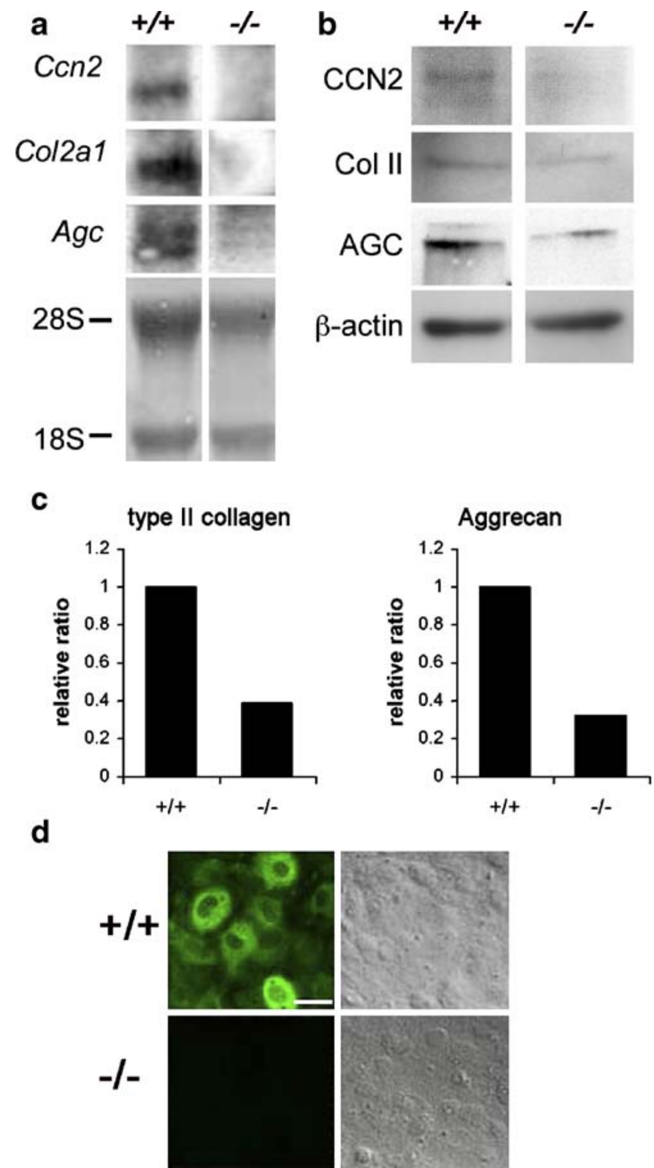

Fig. 2 Effect of CCN2 on chondrocyte proliferation and differentiation. Expression of *Ccn2*, *Col2A1*, and *aggrecan* in chondrocytes. **a** RNA was isolated from confluent cultures of chondrocytes and analyzed by Northern blot. The *bottom panel* shows methylene blue staining for ribosomal RNAs as a loading control. **b** Western blot analysis of CCN2, collagen type II, and aggrecan protein in whole lysates of confluent chondrocytes. The *bottom panel* shows β-actin as a loading control. **c** Densitometric analysis of protein levels of type II collagen (*left panel*) and aggrecan (*right panel*). WT levels are set at 1.0, and the relative ratio of expression in *Ccn2*$^{-/-}$ chondrocytes is indicated. Data shown are from a representative experiment. **d** Immunofluorescence analysis of type II collagen expression. Confocal laser scanning immunofluorescent and corresponding phase contrast images are shown for WT and *Ccn2*$^{-/-}$ chondrocytes. The *bar* represents 30 μm. The Northern, Western, and immunofluorescence analyses were repeated three times with similar results. Representative experiments are shown

lower in *Ccn2*$^{-/-}$ chondrocytes (Fig. 2c). Impaired type II collagen production in *Ccn2*$^{-/-}$ cartilage has not been previously reported. Therefore, to confirm decreased expression of type II collagen, we performed immunofluorescence on WT and *Ccn2*$^{-/-}$ chondrocytes. Figure 2d shows that few *Ccn2*$^{-/-}$ chondrocytes express normal levels of type II collagen. These data are consistent with previous studies showing that exogenous CCN2 enhances

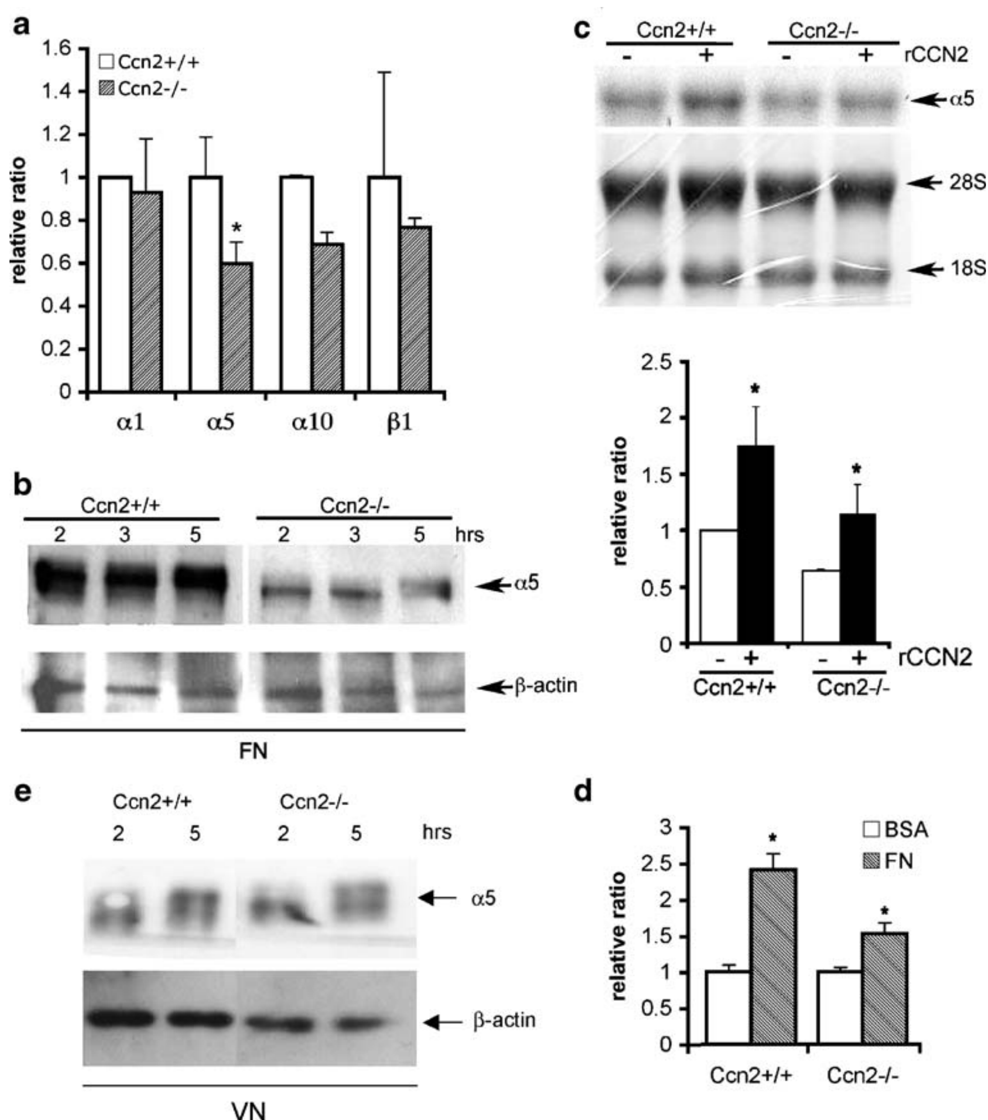

Fig. 3 Downregulation of α5 integrin expression and reduced adhesion of *Ccn2*$^{-/-}$ chondrocytes. **a** Expression of integrins α1, α5, α10, and β1 in WT and *Ccn2*$^{-/-}$ chondrocytes. WT and *Ccn2*$^{-/-}$ chondrocytes were cultured until confluent. Total RNA was collected and quantitative real-time RT-PCR analysis was performed. Data presented are mean and S.D. relative to WT (relative expression=1.0), of two separate reactions using mRNA from two different cultures. The *asterisk* indicates significance at $p<0.05$. **b** Western blot analysis of integrin α5 protein in lysates of WT and *Ccn2*$^{-/-}$ chondrocytes spread on FN for the indicated times. Levels of integrin α5 are decreased in *Ccn2*$^{-/-}$ chondrocytes beginning at the earliest stage monitored. **c** Effect of rCCN2 on the expression of integrin α5 in WT and *Ccn2*$^{-/-}$ chondrocytes. WT and *Ccn2*$^{-/-}$ cells were cultured until confluent. Thereafter, the medium was replaced with a serum-free medium containing rCCN2 at 50 ng/ml, and total RNA was collected 6 h later. Top panel: Northern blot analysis was performed using a probe specific for integrin α5, and methylene blue staining for the ribosomal subunits. *Bottom panel*: The amount of integrin α5 mRNA was determined densitometrically and normalized to 18S-ribosomal RNA. Data presented are mean and S.D. relative to WT using mRNA from two different experiments. The asterisk indicates significance at $p<0.05$. **d** Cell attachment assay. WT and *Ccn2*$^{-/-}$ chondrocytes were inoculated at a density of 5×10^4/well in 96-well plates coated with FN or BSA (each 5 μg/well) overnight. The ordinate shows the relative ratio of adhesion normalized to BSA (=1). Each column represents the mean and S.D. of 10 wells. The asterisks indicate a significant difference ($p<0.05$) between WT and *Ccn2*$^{-/-}$ chondrocytes. **e** Effect of VN on integrin α5 expression. WT and *Ccn2*$^{-/-}$ chondrocytes were plated at a density of 2×10^5/well in 6-well plates coated with VN. Levels of integrin α5 are decreased in *Ccn2*$^{-/-}$ cells planted on FN (Fig. 3b), but are indistinguishable from WT when plated on VN

ECM production in chondrocytes in vitro (Nakanishi et al. 2000). Consistent with the persistence of some type II collagen in protein lysates prepared from *Ccn2*$^{-/-}$ chondrocytes (Fig. 2b), type II collagen can occasionally be detected in *Ccn2*$^{-/-}$ cells by immunohistochemistry (Fig. S1b, Supplementary data). In contrast to the reduced

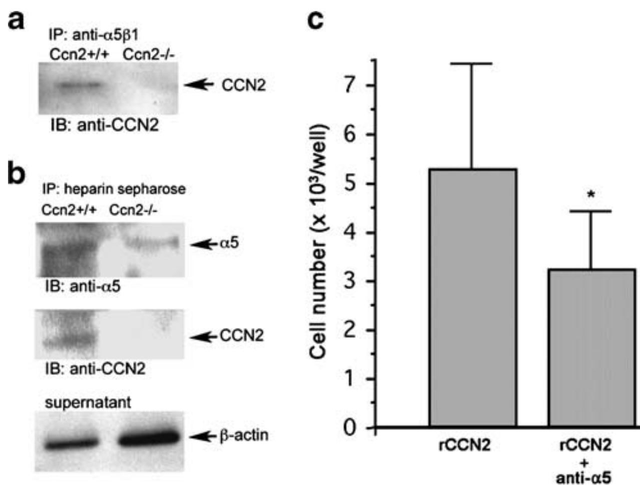

Fig. 4 CCN2 is a ligand for integrin α5 in primary chondrocytes. **a** Complex formation between CCN2 and integrin α5. WT and *Ccn2*$^{-/-}$ chondrocytes were cultured until confluent. Lysates of WT and *Ccn2*$^{-/-}$ chondrocytes were incubated with antibody against integrin α5, and proteins were detected by western blot with an anti-CCN2 antibody. **b** Lysates of WT and *Ccn2*$^{-/-}$ chondrocytes were precipitated with heparin sepharose and integrin α5 and CCN2 were detected by Western blot. **c** Adhesion to rCCN2 is impaired by anti-integrin α5 function-blocking antibodies. Chondrocytes were plated on rCCN2-coated plates treated with or without blocking antibody. The *asterisk* indicates a significant difference ($p<0.05$) between adhesion on cells in the presence or absence of the blocking antibody

expression seen in isolated *Ccn2*$^{-/-}$ chondrocytes, type II collagen levels in intact *Ccn2*$^{-/-}$ growth plates are indistinguishable from those in WT growth plates (Ivkovic et al. 2003). Compensatory mechanisms, possibly involving other CCN family members, are most likely responsible for the ability of *Ccn2*$^{-/-}$ chondrocytes in intact growth plates and in some isolated cells to produce type II collagen. Taken together, the results demonstrate that *Ccn2* is required in chondrocytes for normal expression of multiple cartilage-specific ECM components.

Expression and function of integrin α5 in *Ccn2*$^{-/-}$ mutant chondrocytes

It is well known that interactions of integrins with ECM components are required for cartilage proliferation and differentiation (Loeser 2002). It has also been shown that CCN2 binds several types of integrins in fibroblasts and endothelial cells (Lau and Lam 1999; Brigstock 2003; Babic et al. 1999; Leu et al. 2003; Chen et al. 2004; Hoshijima et al. 2006; Tong and Brigstock 2006; Gao and Brigstock 2005, 2006). Therefore, we examined levels of expression of various integrin subunits in chondrocytes by quantitative real-time PCR analysis. We focused on integrins α1, α5, α10 and β1 because loss-of-function approaches have revealed roles for these subunits in intact

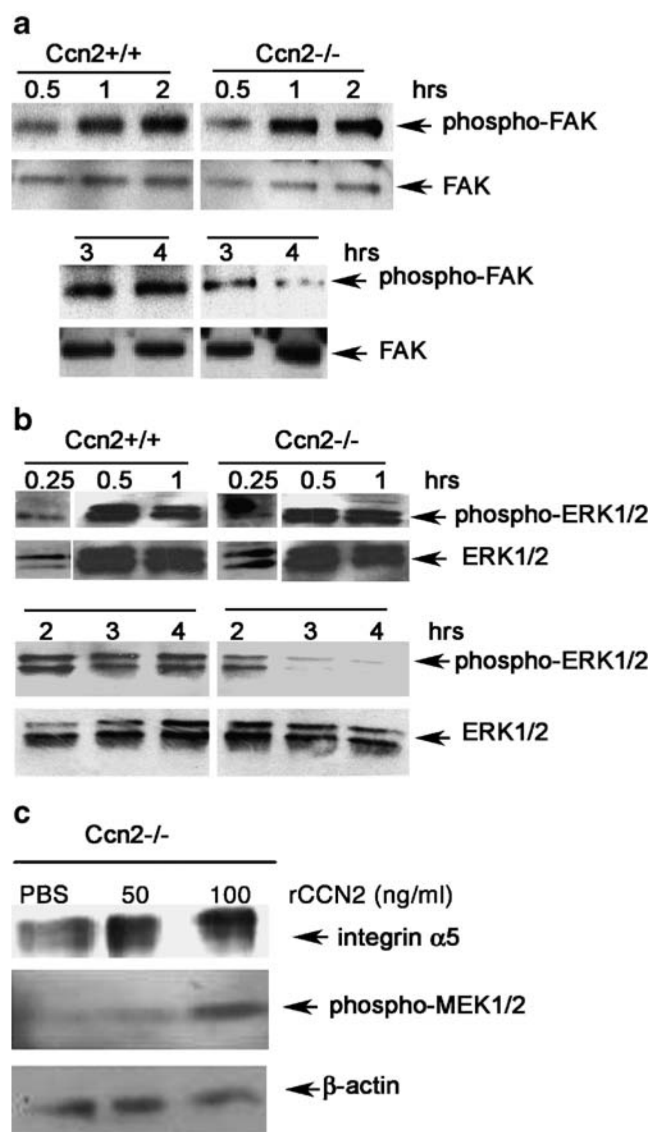

Fig. 5 Western blot analysis of phospho-FAK and phospho-ERK1/2 in WT and *Ccn2* mutant cells. **a** CCN2 is required for maintenance of phospho-FAK activity on FN. WT or *Ccn2*$^{-/-}$ chondrocytes were inoculated on FN (5 μg/well) at 37°C overnight. Cell lysates were collected 0.5, 1, 2, 3, or 4 h after inoculation, and immunoprecipitated with anti-FAK antibody at 4°C overnight. The precipitates were subjected to SDS-PAGE and Western blot was performed with anti-phospho-FAK and anti-FAK antibodies. **b** CCN2 is required for maintenance of pERK1/2 levels of FN. WT or *Ccn2*$^{-/-}$ cells were cultured as above for the indicated times. Immunoprecipitation was performed with anti-ERK1/2 antibody, followed by detection with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. **c** Ability of rCCN2 to rescue pMEK1/2 activity in *Ccn2*$^{-/-}$ chondrocytes plated on FN. Cells were plated on FN as described above, and maintained in the absence or presence of rCCN2 at the indicated concentrations for 4 h. Integrin α5 and pMEK1/2 were detected by Western blot analysis

cartilage in vivo (Aszodi et al. 2003; Garciadiego-Cázares et al. 2004; Zemmyo et al. 2003; Ekholm et al. 2002; Bengtsson et al. 2005). Moreover, exogenous CCN2

upregulates integrin α5 expression in normal rat kidney (NRK) fibroblasts (Frazier et al. 1996), suggesting that *Ccn2*$^{-/-}$ chondrocytes might exhibit reduced expression of this integrin subunit. Indeed, expression of integrin α5 was decreased in *Ccn2*$^{-/-}$ chondrocytes compared to WT cells (Fig. 3a). Expression of integrin α10 showed a trend towards decreased expression in *Ccn2*$^{-/-}$ cells, but the difference did not reach statistical significance. Expression of the other integrins was not obviously different (Fig. 3a). To confirm that integrin α5 protein was decreased, we analyzed protein levels in *Ccn2*$^{-/-}$ chondrocytes plated on FN. Figure 3b shows that integrin α5 protein is expressed at significantly lower levels in *Ccn2*$^{-/-}$ chondrocytes compared to WT cells at all time points monitored, reflecting the decrease observed in mRNA. These results raised the possibility that CCN2 could be directly involved in determining the expression level of integrin α5 in chondrocytes. To investigate this possibility further, we examined the effects of culturing WT and *Ccn2*$^{-/-}$ chondrocytes in the presence of recombinant CCN2 protein (rCCN2). Both WT and *Ccn2*$^{-/-}$ chondrocytes exhibited increased expression of integrin α5 in the presence of rCCN2 (Fig. 3c), suggesting that CCN2 indeed has a positive role in regulating levels of integrin α5 expression. To determine whether the ability of rCCN2 to restore expression of integrin α5 was a direct or indirect effect, we investigated whether rCCN2 could increase integrin α5 mRNA levels in WT chondrocytes in the presence of cycloheximide. Under these conditions, no increased integrin α5 expression was observed (data not shown), indicating that new protein synthesis is required for rCCN2 to upregulate integrin α5 levels in chondrocytes.

Integrin α5β1 is the major receptor for FN, and a major receptor for COMP (cartilage oligomeric matrix protein) in chondrocytes (Chen et al. 2005). Therefore, we investigated whether *Ccn2*$^{-/-}$ chondrocytes exhibit impaired ability to bind to FN. Figure 3d shows that, consistent with decreased integrin α5 expression, adhesion of *Ccn2*$^{-/-}$ chondrocytes to FN was reduced. The results described above suggest that *Ccn2*$^{-/-}$ cells are defective in adhesion to FN, at least in part because of decreased α5β1 expression. It has been shown previously that FN-mediated adhesive signaling maintains integrin α5 expression (Huang et al. 1994; Larouche et al. 2000), suggesting that the impaired adhesion to FN seen in *Ccn2*$^{-/-}$ cells can be both a cause, and a consequence of, reduced integrin α5 expression, and that CCN2 plays a direct role in the positive feedback loop by which FN binding to α5β1 integrin upregulates integrin α5 expression. If so, integrin α5 levels should be similar in WT and *Ccn2*$^{-/-}$ cells plated on VN, which does not engage integrin α5β1. As previously described, *Ccn2*$^{-/-}$ chondrocytes exhibit impaired expression of integrin α5 on FN (Fig. 3b); however, α5 levels were indistinguishable between WT and *Ccn2*$^{-/-}$ cells when they were plated on VN (Fig. 3e). These results imply that the decreased adhesion exhibited by *Ccn2*$^{-/-}$ chondrocytes on FN and under normal conditions, where they produce their own matrix, is mediated by α5β1 integrins, and that this decreased adhesion is responsible for the decreased integrin α5 expression.

CCN2 physically interacts with α5β1 in fibroblasts (Chen et al. 2004), hepatic and pancreatic stellate cells (Gao and Brigstock 2005, 2006) and in a chondrosarcoma-derived cell line (Hoshijima et al. 2006). Therefore, we tested whether CCN2 physically interacts with α5β1 integrin in primary chondrocytes. Integrin α5 was precipitated from WT and *Ccn2*$^{-/-}$ chondrocyte lysates, followed by Western blotting with antibodies against CCN2. Figure 4a shows that CCN2 can be detected in integrin α5 precipitates from WT chondrocytes, but not from *Ccn2*$^{-/-}$ cells, demonstrating that CCN2 binds integrin α5 in primary chondrocytes. In the absence of an antibody that precipitates CCN2, we used heparin sepharose to precipitate lysates, because CCN2 binds heparin strongly. As shown in Fig. 4b, binding of integrin α5 to heparin was observed in cell lysates from both WT and *Ccn2*$^{-/-}$ chondrocytes; however, the intensity of the band was decreased in lysates from mutant cells, consistent with the decreased expression of α5 integrin. When these immunoprecipitates were immunoblotted to detect CCN2, this protein was clearly present in WT chondrocytes, but absent in *Ccn2*$^{-/-}$ chondrocytes. These data demonstrate that

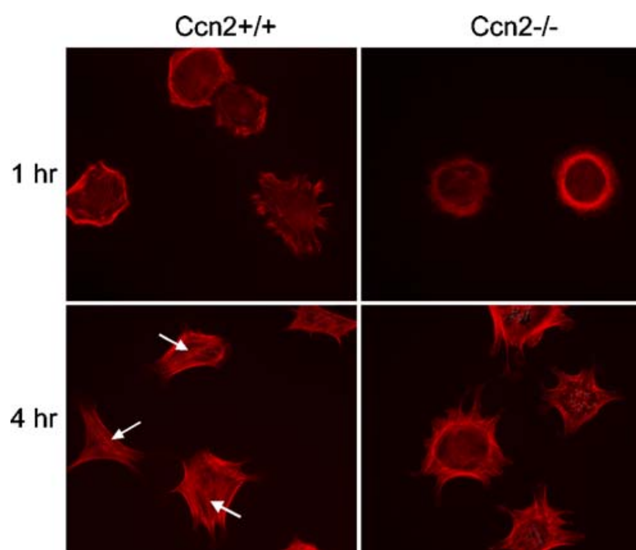

Fig. 6 Immunofluorescence analysis of the actin cytoskeleton in WT or *Ccn2*$^{-/-}$ chondrocytes on FN. WT and *Ccn2*$^{-/-}$ chondrocytes were spread on FN coated with slides for 1 h or 4 h. The cultures were fixed with 3.5% formaldehyde, permeabilized with 0.1% NP-40 in PBS, and stained with phalloidin conjugated to alexa fluor-555 to visualize actin filaments. Arrows indicate stress fibers

CCN2 is an endogenous ligand for integrin α5 in chondrocytes.

In order to test the potential significance of integrin α5 as a receptor for CCN2 in chondrocytes, we examined the effect of blocking antibodies to integrin α5 on the ability of these cells to adhere to exogenous CCN2. As shown in Fig. 4c, adhesion of primary chondrocytes to rCCN2 is impaired by the integrin α5 blocking antibody. This result indicates that integrin α5 is a receptor for CCN2 in primary chondrocytes. Taken together, the data thus suggest that CCN2 regulates chondrocyte function both as a ligand for integrin α5, and as a regulator of α5 expression.

CCN2 maintains phosphorylation of FAK and ERK1/2 in chondrocytes

Because expression of integrin α5 and adhesion to FN were decreased in $Ccn2^{-/-}$ chondrocytes, and CCN2 is known to activate focal adhesion kinase (FAK) (Chen et al. 2001), we tested whether adhesive signaling was also impaired in *Ccn2* deficient cells. Ligation of β1 integrins results in phosphorylation of FAK as it is recruited to focal adhesions, leading to activation of the downstream extracellular signal-regulated kinase (ERK)1/2 pathway (Schlaepfer et al. 1999). WT and $Ccn2^{-/-}$ chondrocytes

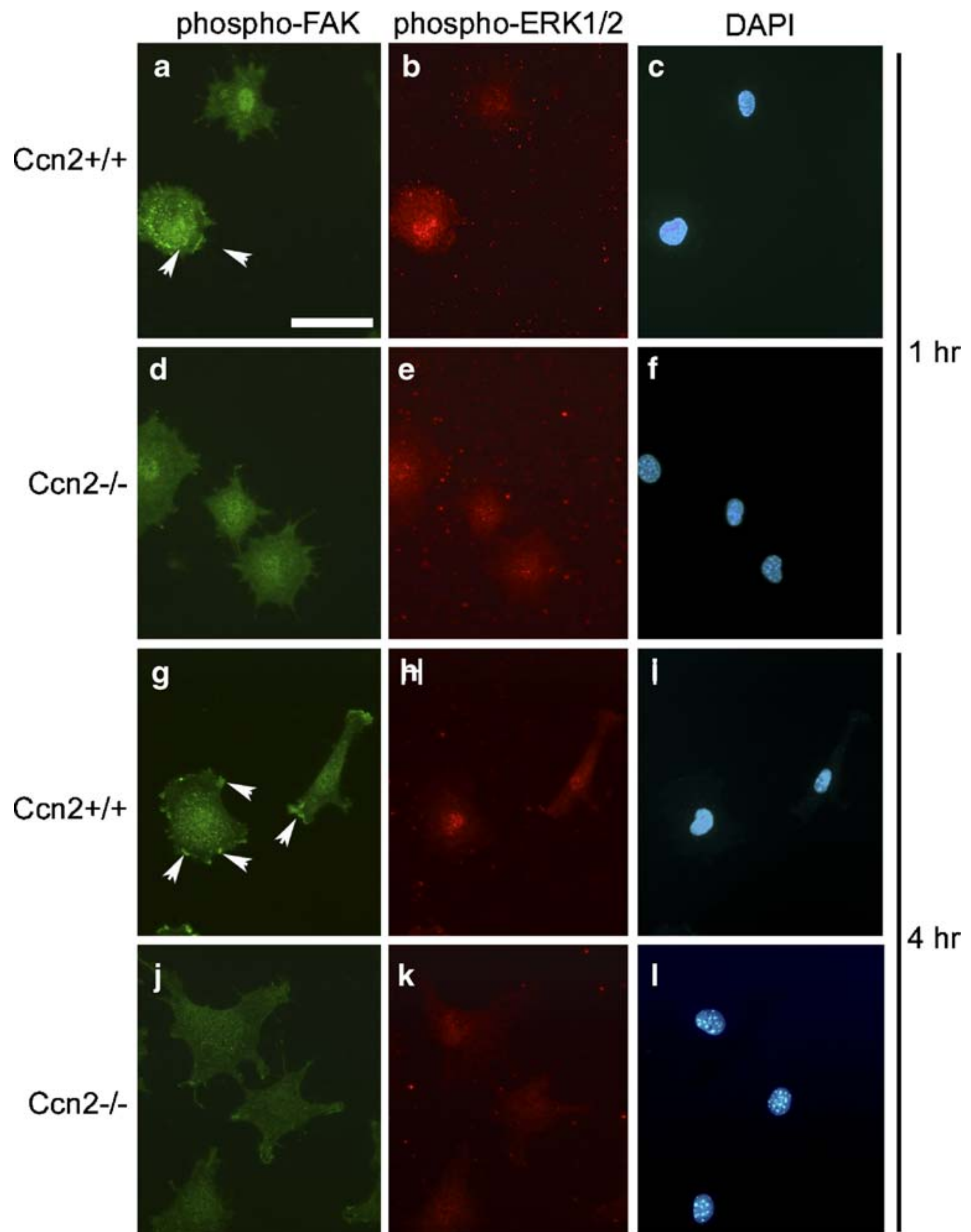

Fig. 7 Immunofluorescence analysis of phospho-FAK and phospho-ERK1/2 in WT or $Ccn2^{-/-}$ chondrocytes. WT (**a**–**c**, **g**–**i**) and $Ccn2^{-/-}$ (**d**–**f**, **j**–**l**) chondrocytes were spread on FN coated slides for 1 h (**a**–**f**) or 4 h (**g**–**l**). Localization of phospho-FAK (**a**, **d**, **g**, **j**) and phospho-ERK1/2 (**b**, **e**, **h**, **k**) was visualized by indirect immunofluorescence. Microphotographs of a single field stained with anti-phospho-FAK (*green*) and phospho–ERK1/2 (*red*) antibodies and with DAPI (**c**, **f**, **i**, **l**). The *arrows* indicate focal adhesions (**a** and **g**). *Bar* represents 50 μm

were allowed to adhere to FN for various times before immunoprecipitation analysis with antibodies specific for total FAK or the phosphorylated (activated) form. At early time points (up to 2 h) levels of FAK phosphorylation were similar in WT and *Ccn2*$^{-/-}$ chondrocytes. However, at later times the degree of phosphorylation decreased considerably in the *Ccn2*$^{-/-}$ chondrocytes (Fig. 5a). Similar results were obtained when levels of ERK1/2 phosphorylation were examined (Fig. 5b). To determine whether the decreased expression of integrin $\alpha5$ may contribute to the reduced adhesive signaling in *Ccn2*$^{-/-}$ chondrocytes, we tested whether rCCN2 could restore both integrin $\alpha5$ and phospho-MEK1/2 levels. As shown in Fig. 5c, rCCN2 at 50 ng/ml and 100 ng/ml increased integrin $\alpha5$ levels on chondrocytes plated on FN (Fig. 5c). Moreover, increased phospho-MEK1/2 levels were observed in the presence of 100 ng/ml rCCN2 (Fig. 5c). The fact that integrin $\alpha5$ levels are increased by 50 ng/ml rCCN2, but elevation of phospho-MEK1/2 levels requires a higher concentration (100 ng/ml) of rCCN2 raises the likelihood that the ability of CCN2 to upregulate integrin $\alpha5$ expression is required, but that rCCN2 also mediates additional activities to maintain adhesive signaling in chondrocytes.

Subcellular localization of actin, phosho-FAK, and phospho-ERK1/2 in *Ccn2*$^{-/-}$ chondrocytes

The role of CCN2 in adhesive signaling in chondrocytes was further investigated by indirect immunofluorescence. WT and *Ccn2*$^{-/-}$ chondrocytes were seeded on FN for 1 or 4 h before being fixed in formaldehyde, and actin filaments visualized with phalloidin conjugated to a fluorescent marker (Fig. 6). The majority of actin is present close to the cell periphery in WT and *Ccn2*$^{-/-}$ chondrocytes seeded for 1 h on FN. After 4 h there are differences between WT and *Ccn2*$^{-/-}$ chondrocytes; in WT chondrocytes stress fibers are evident, whereas in *Ccn2*$^{-/-}$ cells the majority of the actin remains close the cell periphery, consistent with defective adhesive signaling.

To investigate this possibility further, we subjected WT and *Ccn2*$^{-/-}$ chondrocytes to double immunostaining with anti-phospho-FAK and anti-phospho-ERK1/2 antibodies. Phospho-FAK is a marker for focal adhesions (e.g., Almeida et al. 2000; Ruest et al. 2000; Holinstat et al. 2006; Zaidel-Bar et al. 2007). As shown in Fig. 7, immunoreactivity for phospho-FAK was detected in the nucleus in both WT and *Ccn2*$^{-/-}$ chondrocytes after 1 h on FN, and Western blot analysis revealed similar overall levels of phospho-FAK (Fig. 5a). However, staining in focal adhesions was seen only in WT cells (Fig. 7a,d; arrows). In WT cells, the presence of phospho-FAK in the nucleus and in focal adhesions persisted after 4 h on FN (Fig. 7g). However, phospho-FAK could not be detected in the nucleus or in focal adhesions of *Ccn2*$^{-/-}$ chondrocytes after 4 h on FN (Fig. 7j). Indirect immunofluorescence for vinculin and phospho-tyrosine, additional markers for focal adhesions (Hamadi et al. 2005; Ballestrem et al. 2006; Robels and Gomez 2006; Sawada et al. 2006) confirmed that focal adhesion formation is defective in *Ccn2*$^{-/-}$ chondrocytes (Fig. 8). In the case of phospho-ERK1/2, immunoreactivity was seen in the nucleus and cytoplasm of WT chondrocytes after 1 h on FN (Fig. 7b), and persisted in the nucleus after 4 h (Fig. 7h). However, both nuclear and cytoplasmic staining of phosph-ERK1/2 was reduced in *Ccn2*$^{-/-}$ chondrocytes after 1 and 4 h on FN (Fig. 7e,k).

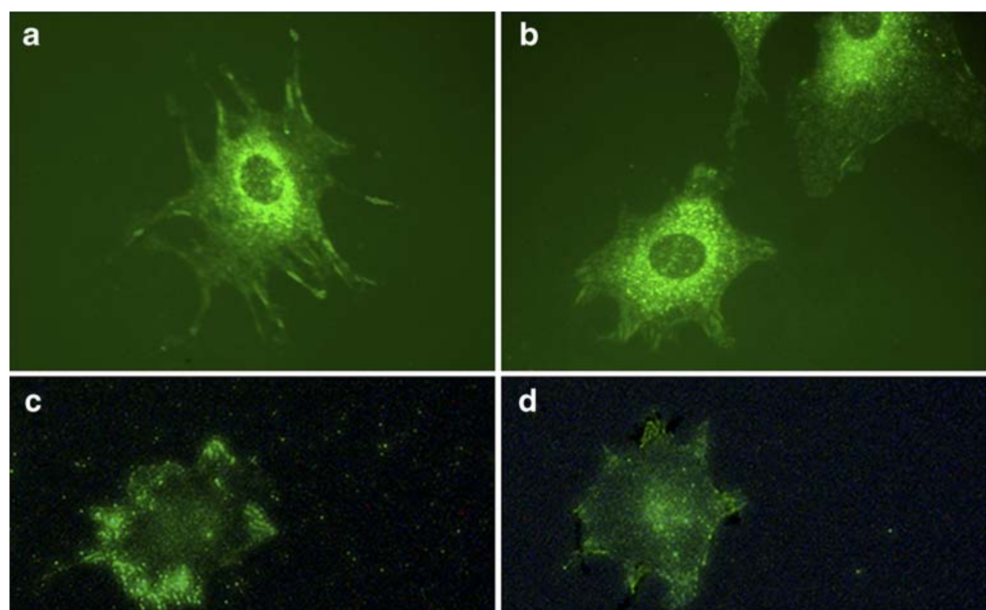

Fig. 8 Focal adhesion formation in WT and *Ccn2*$^{-/-}$ chondrocytes. **a**, **b** WT and *Ccn2*$^{-/-}$ chondrocyte, respectively, plated on FN for 4 h followed by indirect immunofluorescence analysis of vinculin expression. Focal adhesions are seen at the periphery of the WT cell but not the mutant cell. **c**, **d** WT and *Ccn2*$^{-/-}$ chondrocyte, respectively, stained with anti-phosphotyrosine antibody (PY-20)

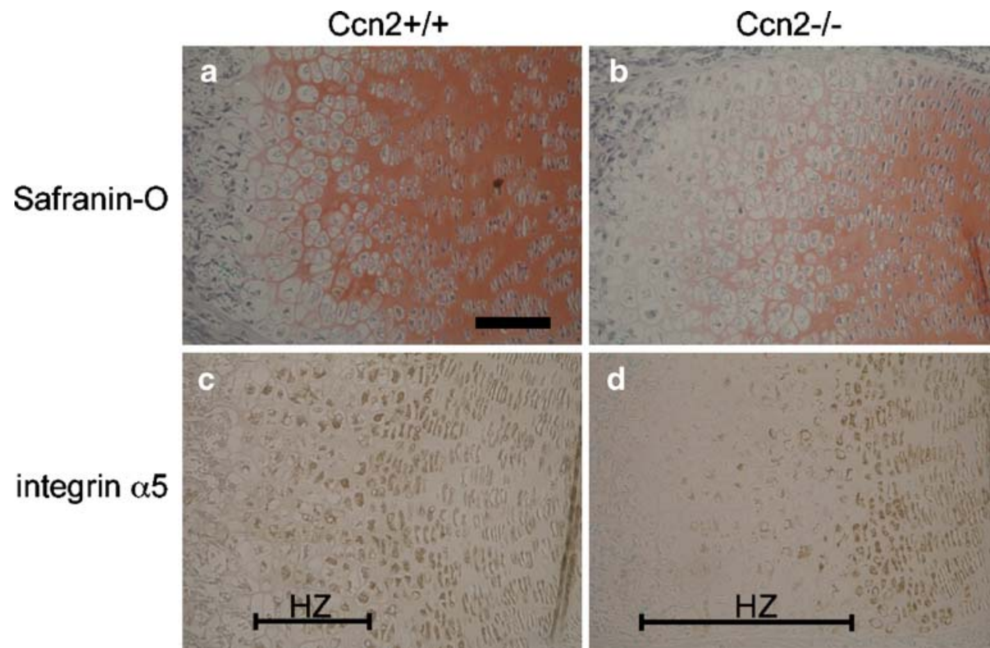

Fig. 9 Immunolocalization of $\alpha5\beta1$ integrin in sections of growth plate cartilage of WT and *Ccn2*$^{-/-}$ mice. Sections of growth plates of E18.5 femurs at identical levels in WT and *Ccn2*$^{-/-}$ littermates were stained with safranin-O (**a**, **b**), and reacted with anti-$\alpha5\beta1$ integrin (**c**, **d**). The primary antibodies were visualized by immunoperoxidase, and then the sections were counterstained with methyl green. $\alpha5\beta1$ integrin was localized throughout the growth plate, with highest levels of expression in the hypertrophic zone (HZ) of the WT growth plate. Lower levels of expression are seen in the hypertrophic zone in the mutant growth plate. *Bar* represents 100 μm

These findings are consistent with the results in Fig. 5 and indicate that cell signaling through integrin receptors for FN via phospho-FAK and phospho-ERK1/2 is maintained in WT, but not in *Ccn2*$^{-/-}$ chondrocytes.

Immunolocalization of integrin α5 in WT and *Ccn2*$^{-/-}$ growth plates

Our data suggest that CCN2 is essential for ECM production and both induces the expression of integrin α5, and binds to it. To determine whether decreased expression of ECM components and/or integrin α5 might contribute to the chondrodysplaisa observed in *Ccn2*$^{-/-}$ mice, we examined proteoglycan production and the localization of integrin α5 in endochondral bone. As shown in Fig. 9, an enlarged hypertrophic zone was seen in the growth plate in *Ccn2*$^{-/-}$ mice compared to WT littermates (panels a and b), consistent with previous studies (Ivkovic et al. 2003). Safranin-O staining revealed reduced levels of proteoglycans in cartilage throughout *Ccn2*$^{-/-}$ growth plates (Fig. 9a and b). This finding is consistent with the results shown in Fig. 2 and with previous studies indicating that aggrecan levels are reduced in *Ccn2*$^{-/-}$ growth plates (Ivkovic et al. 2003). In accordance with previous studies (Loeser 2002), immunoreactivity for integrin α5 was detected throughout the WT growth plate, with highest levels in the prehypertrophic and hypertrophic zones (Fig. 9c). This pattern of expression corresponds closely with that of CCN2 in the growth plate; CCN2 is expressed at low levels in proliferating chondrocytes and at much higher levels in the hypertrophic zone (Ivkovic et al. 2003; Nishida et al. 2002). The pattern of integrin α5 expression is significantly reduced in *Ccn2*$^{-/-}$ mice, with the greatest reduction in the hypertrophic zone chondrocytes (Fig. 9 compare panels c and d). The finding that integrin α5 expression is reduced in *Ccn2*$^{-/-}$ growth plates, and this reduction is most evident in the hypertrophic zone, which normally expresses the highest levels of CCN2, strongly suggests that integrin α5 is a major mediator of the effects of CCN2 *in vivo* in chondrocytes.

Discussion

Previous studies have shown that CCN2 is an essential regulator of chondrogenesis (Ivkovic et al. 2003). Chondrocyte proliferation, ECM composition, matrix remodeling and growth plate angiogenesis are all impaired in *Ccn2*$^{-/-}$ growth plates. The mechanisms by which loss of CCN2 leads to these defects are unknown. We have investigated potential mechanisms of action of CCN2 in chondrocytes in this report.

We find that chondrocytes isolated from *Ccn2*$^{-/-}$ mice exhibit impaired type II collagen and aggrecan expression, matrix synthesis, and proliferation, consistent with and extending our previous findings in vivo (Ivkovic et al. 2003). Moreover, we find that CCN2 is an essential regulator of integrin α5 expression and function in chondrocytes. We show here that rCCN2 induces integrin α5 expression, and that endogenous CCN2 is required for normal levels of integrin α5 expression in chondrocytes.

Recently, it was reported that blocking α5β1 integrin function *in vivo* inhibits pre-hypertrophic chondrocyte differentiation (Garciadiego-Cázares et al. 2004). In this study, we show that decreased expression of integrin α5 in *Ccn2*$^{-/-}$ chondrocytes correlates with defective chondrocyte differentiation and ECM production *in vitro* and *in vivo*. Interestingly, the expression pattern of integrin α5 in the cartilage strongly resembles that previously shown for CCN2 (Nakanishi et al. 2000; Ivkovic et al. 2003; Nishida et al. 2002). This strongly suggests a potential role for CCN2 as an endogenous regulator of integrin α5 expression. However, CCN2 cannot be the only regulator of integrin α5 expression in cartilage because it is clearly present in *Ccn2*$^{-/-}$ growth plates (Fig. 9), and isolated *Ccn2*$^{-/-}$ primary chondrocytes retain low levels of integrin α5 expression (Fig. 3a,b). It is conceivable that the related family member CCN1 (CYR61), which is expressed in proliferative and pre-hypertrophic chondrocytes (Wong et al. 1997), can compensate at least in part for loss of *Ccn2* and is responsible for the residual level of integrin α5 expression. We conclude that regulation of integrin α5 expression represents one, but not the only, mechanism underlying the requirement for CCN2 in chondrogenesis. That additional mechanisms of CCN2 action are responsible for the defects in adhesive signaling in *Ccn2* mutant chondrocytes is further suggested by the finding that rCCN2 at 50 ng/ml can rescue integrin α5 expression, but not phospho-MEK1/2 levels (Fig. 5).

We have shown that CCN2 is present in a complex with integrin α5 in chondrocytes. Our data support the hypothesis that CCN2 is required for efficient signaling by integrin α5 in chondrocytes. Although initial phosphorylation of FAK and ERK1/2 appears to be normal in *Ccn2*$^{-/-}$ chondrocytes, the signal is transient compared to the persistence of these signals in WT cells. The basis for this inability of mutant chondrocytes to maintain phospho-FAK and phospho-ERK1/2 at normal levels is currently unknown, but warrants investigation in the future. Immunofluorescence studies verified defective focal adhesion formation in *Ccn2*$^{-/-}$ chondrocytes. In addition, there are significant phenotypic similarities in the defects observed in *Ccn2*$^{-/-}$ growth plates and those in mice that lack expression of integrin β1 (Aszodi et al. 2003). Taken together, these data are consistent with the hypothesis that CCN2 is required for integrin-mediated signaling in chondrocytes, particularly through integrin α5β1. Our data show that CCN2 not only induces integrin α5 expression (Fig. 3c) and is required for normal expression levels in vivo (Fig. 9), but that integrin α5 also interacts directly with CCN2 in chondrocytes (Fig. 4).

It has been shown that expression of integrin α5 is upregulated by exogenous CCN2 in fibroblasts (Frazier et al. 1996; Weston et al. 2003). Recently, Chen *et al.* investigated cell adhesion to fibronectin using *Ccn2*$^{-/-}$ embryonic fibroblasts, and showed that loss of endogenous CCN2 resulted in impaired spreading on fibronectin, delayed stress fiber formation, and reduced FAK and ERK1/2 phosphorylation (Chen et al. 2004). Interestingly, in contrast to our finding that integrin α5 mRNA and protein levels are reduced in *Ccn2*$^{-/-}$ chondrocytes (Fig. 3a,b), no such effect on α5 integrin transcription was observed in *Ccn2*$^{-/-}$ fibroblasts (Chen et al. 2004). However, our results are consistent with other reports that α5 integrin signaling induces α5 integrin transcription (Huang et al. 1994). The observation that FAK and ERK1/2 cannot be maintained in *Ccn2*$^{-/-}$ chondrocytes (Figs. 5 and 7) provides strong support for the hypothesis that the chondrodysplasia observed in *Ccn2*$^{-/-}$ growth plates is due at least in part to defective integrin-ECM interactions.

In conclusion, CCN2 is an essential regulator of ECM expression and both the expression and signaling properties of integrin α5; moreover CCN2 mediates signaling through the activation of ERK1/2 via integrin signaling in chondrocytes. The phenotypic correspondence between β1 integrin-deficient mice (Aszodi et al. 2003) and *Ccn2*$^{-/-}$ mice (Ivkovic et al. 2003) is consistent with the possibility that a primary mode of action for CCN2 is through its ability to regulate integrin signaling.

CCN2 has been proposed to bind directly to a variety of growth factors, including Transforming growth factor (TGF)-β, Bone morphogenetic proteins (BMPs), and vascular endothelial growth factor (VEGF), all of which have profound effects on the growth plate (Serra et al. 1997; Inoki et al. 2002; Abreu et al. 2002; Zelzer et al. 2002; Yoon et al. 2005, 2006; Baffi et al. 2006). Future studies will be required to determine the physiological relevance of these activities of CCN2, to determine whether CCN2 exerts overlapping functions with other CCN family members in cartilage, and to detect additional functions for CCN2 in other aspects of disease and development.

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Statement of competing interests The authors declare that they have no competing interests.

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