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Authors
Hall-Glenn, Faith
Aivazi, Armen
Akopyan, Lusi
et al.

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CCN2/CTGF is required for matrix organization and to protect growth plate chondrocytes from cellular stress

Faith Hall-Glenn · Armen Aivazi · Lusi Akopyan · Jessica R. Ong · Ruth R. Baxter · Paul D. Benya · Roel Goldschmeding · Frans A. van Nieuwenhoven · Ernst B. Hunziker · Karen M. Lyons

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Abstract CCN2 (connective tissue growth factor (CTGF/CCN2)) is a matricellular protein that utilizes integrins to regulate cell proliferation, migration and survival. The loss of CCN2 leads to perinatal lethality resulting from a severe chondrodysplasia. Upon closer inspection of Ccn2 mutant mice, we observed defects in extracellular matrix (ECM) organization and hypothesized that the severe chondrodysplasia caused by loss of CCN2 might be associated with defective chondrocyte survival. Ccn2 mutant growth plate chondrocytes exhibited enlarged endoplasmic reticula (ER), suggesting cellular stress. Immunofluorescence analysis confirmed elevated stress in Ccn2 mutants, with reduced stress observed in Ccn2 overexpressing transgenic mice. In vitro studies revealed that Ccn2 is a stress responsive gene in chondrocytes. The elevated stress observed in Ccn2−/− chondrocytes is direct and mediated in part through integrin α5. The expression of the survival marker NFκB and components of the autophagy pathway were decreased in Ccn2 mutant growth plates, suggesting that CCN2 may be involved in mediating chondrocyte survival. These data demonstrate that absence of a matricellular protein can result in increased cellular stress and highlight a novel protective role for CCN2 in chondrocyte survival. The severe chondrodysplasia caused by the loss of CCN2 may be due to increased chondrocyte stress and defective activation of autophagy pathways, leading to decreased cellular survival. These effects may be mediated through nuclear factor κB (NFκB) as part of a CCN2/integrin/NFκB signaling cascade.

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F. Hall-Glenn · A. Aivazi · L. Akopyan · J. R. Ong · K. M. Lyons
Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, Los Angeles, CA 90095, USA

R. R. Baxter · P. D. Benya · K. M. Lyons
Department of Orthopaedic Surgery, University of California, Los Angeles, Los Angeles, CA 90095, USA

R. Goldschmeding
Department of Pathology, University of Utrecht, Utrecht, Netherlands

F. A. van Nieuwenhoven
Department of Physiology, Maastricht University, Maastricht, Netherlands

E. B. Hunziker
Centre of Regenerative Medicine for Skeletal Tissues, Department of Clinical Research, University of Bern, Bern, Switzerland

K. M. Lyons
Department of Orthopaedic Surgery, Orthopaedic Hospital Research Center, University of California Los Angeles, 615 Charles E Young Drive South 501, Los Angeles, CA 90095, USA
e-mail: klyons@mednet.ucla.edu

Present Address:
A. Aivazi
School of Medicine, Stony Brook University, East Loop Road, Stony Brook, NY, USA

Present Address:
L. Akopyan
Western University of Health Sciences College of Pharmacy, 309 E. Second street, Pomona, CA, USA
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Introduction

Chondrocytes secrete large amounts of extracellular matrix (ECM) as they differentiate. Disruptions in this process often result in chondrodysplasias, a diverse group of congenital birth defects characterized by short stature and skeletal deformity. The most common causes of chondrodysplasias are mutations that lead to structural alterations in genes that encode ECM proteins. These alterations prevent proper protein folding and maturation, leading to their retention in the endoplasmic reticulum (ER), and/or the incorporation of the mutated product into the ECM, interfering with matrix structure and function (Bateman et al. 2009).

ER enlargement is a hallmark of defective protein folding and cellular stress. ER and other forms of cellular stress activate the Unfolded Protein Response (UPR), an adaptive mechanism to restore cell homeostasis and viability (Rasheva and Domingos 2009). The UPR ameliorates cellular stress by attenuating protein synthesis and increasing chaperone protein levels to facilitate correct protein folding and clearing of misfolded protein aggregates. The glucose-regulated protein 78 (GRP78) also known as BiP, is a resident ER chaperone and the main initiator of the UPR (Bertolotti et al. 2000). BiP activates UPR effector molecules that function to restore cellular homeostasis. ER and other forms of cellular stress also activate anti-oxidative stress genes, which promote cell survival; however, prolonged stress can lead to activation of CCAAT/enhancer-binding protein-homologous protein (CHOP) and caspases to induce cell death (Rasheva and Domingos 2009). Although ER sensing of unfolded proteins is the most common inducer of ER stress, the UPR is activated by multiple stress pathways, including hypoxia and ECM detachment (Avivar-Valderas et al. 2011; Walter and Ron 2011). Stress pathways are major players in multiple pathologies including osteoarthritis (Rasheva and Domingos 2009) and identifying proteins involved in cellular stress pathways may provide new therapeutic targets.

Nuclear factor κB (NFκB/p65/reIA) is a transcription factor that plays pivotal roles in cell proliferation, differentiation and survival (Marcus et al. 2010). ER stress activates NFκB via tumor necrosis factor-α (TNF-α) receptor associated factor 2 (TRAF2) and inositol requiring enzyme 1 (IRE1) in vitro (Kaneko et al. 2003). The role of NFκB activation in various forms of cellular stress is unclear, but several studies show that NFκB exerts pro-survival functions (Nozaki et al. 2001; Sirabella et al. 2009). In the growth plate, NFκB is essential for chondrocyte viability (Park et al. 2007; Wu et al. 2007). However, the mechanisms that control NFκB activity in the growth plate are unknown.

Autophagy is a lysosomal degradation pathway used to recycle cellular components and activate cell survival under conditions of starvation and stress (Mehrpour et al. 2010). Autophagy is essential for maintaining ER homeostasis, and evidence indicates that during prolonged ER stress, autophagy is activated to assist in the removal of misfolded proteins (Li et al. 2008). Crosstalk between cellular stress pathways, autophagy and NFκB-mediated cellular survival has been confirmed previously (Copetti et al. 2009; Sirabella et al. 2009), but whether such crosstalk occurs in chondrocytes is unknown.

The term “matricellular” was coined to encompass a structurally diverse group of proteins that reside in the ECM, but serve no structural functions (Bornstein 2009). Members of the CCN (Cyr61/CTGF/Nov) family of matricellular proteins are critical regulators of cell-ECM interactions. The six members of the CCN family contain four conserved domains that mediate interactions with integrins, growth factors, and ECM components including heparin sulfate proteoglycans, fibronectin and aggrecan (Jun and Lau 2011). Connective tissue growth factor (CTGF/CCN2) is best known for its role in fibrosis, where its overexpression exacerbates excess collagen deposition in multiple organs (Brigstock 2010). CCN2 overexpression is involved in many other pathological processes, such as atherosclerosis, osteoarthritis and cancer (Chen and Lau 2009).

Although most studies have focused on the mechanisms by which CCN2 overexpression leads to pathological changes, CCN2 is indispensable for endochondral bone formation. Global loss of CCN2 in mice results in severe chondrodysplasia and lethality at birth (Ivkovic et al. 2003). This phenotype is accompanied by a significant decrease in chondrocyte proliferation, delayed chondrocyte differentiation, impaired ECM production and insufficient vascular invasion (Hall-Glenn et al. 2012; Ivkovic et al. 2003).

CCN2 induces NFκB activity in ATDC5 chondrocytic cells through integrin αβ3-mediated mechanisms to enhance migration (Tan et al. 2009). Reciprocally, the CCN2 promoter contains an NFκB responsive element that activates CCN2 expression during the mechanical stretch response in smooth muscle cells (Chaqour et al. 2006). These studies raise the possibility that CCN2 may regulate NFκB in the growth plate. Given the essential role of NFκB in chondrocyte survival, and evidence that CCN2 induces its expression and activity in multiple cell types, we investigated whether CCN2 plays a role in survival of growth plate chondrocytes. We show that CCN2 is essential for chondrocyte survival, protecting growth plate chondrocytes from pro-apoptotic pathways activated by cellular stress, and promoting chondrocyte survival. We provide evidence
consistent with the possibility that CCN2 enhances chondrocyte survival through activation of NFκB and autophagy. Additionally, we provide evidence that these effects are mediated in part through the ability of CCN2 to engage integrins.

Materials and methods

Mouse strains and cell lines Ccn2 knockout mice were generated, genotyped and maintained as described (Ivkovic et al. 2003). Ccn2−/− embryos were obtained from timed heterozygote matings, with embryonic day (E) 0.5 representing the detection of a post-copulatory plug.

CCN2 floxed inducible transgenic mice (Ccn2fxTg) were generated by pronuclear injection as described (Araki et al. 1995; Voncken 2011). These mice were crossed to Col2a1Cre mice (Ovchinnikov et al. 2000) to induce overexpression of CCN2 in chondrocytes. Genotyping was performed on DNA isolated from tail biopsies with the following primers: Forward: 5′-TCTTCTGCAATTGCCGTCC-3′; Reverse: 5′-AATGTGTCCTCCAGTCGGTAG-3′. Mouse embryonic fibroblasts (MEFs) from Ccn2fxTg embryos were isolated and cultured as described (Lengner et al. 2004). MEFs were infected for 24 hours with adenoviral Cre-recombinase (Ad-Cre) and empty vector controls (Ad-CNT) (University of Iowa Gene Transfer Vector Core) at a multiplicity of infection of 300. RNA was isolated using Qiagen RNeasy Kit, and quantitative RT-PCR (qRT-PCR) was used to quantify relative CCN2 expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described (Kawaki et al. 2008).

Experiments on mice were performed with four sets of WT and Ccn2−/− littermates at E16.5, E18.5 and postnatal day (P) 0 (N=8 per genotype). Animals were treated in accordance with the National Institutes of Health guidelines for care and use of animals, and approved by the UCLA Institutional Animal Care and Use Committee.

Transmission electron microscopy WT and Ccn2−/− E18.5 hindlimbs (N=3 per genotype) were prepared for electron microscopy as previously described (Szafranski et al. 2004). The ultrastructural analysis was performed at the Center of Regenerative Medicine for Skeletal Tissues (Department of Clinical Research, University of Bern), in Bern, Switzerland.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays TUNEL assays (Roche) were performed on WT and Ccn2−/− growth plates according to the manufacturer’s protocol. Cellular counts of chondrocytes were quantitated and normalized to total DAPI counts.

Immunofluorescent staining Embryos were fixed and embedded as described (Ivkovic et al. 2003). 7 µm sections were generated using a Lecia GM40 Microtome. For fluorescent staining, Tyramide Signal Amplification was performed according to the manufacturer’s protocol (Invitrogen). The following antibodies were used: CCN2 L-20 (1:1,000; Santa Cruz Biotechnology), BiP (1:500; Cell Signaling), CHOP (1:400; Cell Signaling), Calnexin (1:300; Chemicon), NFXB/RelA/p65 (1:400; Cell Signaling), Apg/Atg12 (1:400; Chemicon) and Apg8b/LC3 (1:200; Abgent). Secondary antibodies were conjugated with AlexaFluor-594 and 488 (Invitrogen). Sections were counterstained with DAPI (Sigma) and mounted with Prolong Gold reagent (Sigma). Immunofluorescence was visualized on an Olympus Bx60 Microscope.

Sternal chondrocyte isolation E16.5 sterna were isolated and pooled by genotype into conical tubes. Garnet bead mix (Invitrogen) was added to 15 ml of HEPES (Sigma) buffered DMEM (HDMEM) (GIBCO). The connective tissue from the sterna was removed by mechanical shaking for 30 min. The media was aspirated, and 0.03 % bacterial collagenase (Chlostridium; Sigma) in HDMEM was added. Additional shaking was performed for 10 min. Cleared sterna were further digested in 0.01 % collagenase overnight. The following day, chondrocytes were filtered (70 µm Fisher) and plated.

Alginate chondron cultures Isolated sternal chondrocytes were suspended in 1.1 % sodium alginate in 1X Phosphate Buffered Saline (PBS) (Sigma) (Lee et al. 1997). The cell/alginate suspension was extruded drop-wise into 0.1 M Calcium Chloride (Sigma) and beads were polymerized at room temperature for 10–15 min. The beads were then washed with 1X PBS, transferred to T25 flasks and incubated in differentiation media containing 50 ng/ml ascorbic acid (Sigma). After 7–10 days in culture, the chondrons were released using 10 mM sodium citrate (Sigma). Chondron clusters were plated using Cell-tak adhesive (BD Biosciences) and cultured for 1–3 days for immunofluorescence (Hamamura et al. 2009).

Thapsigargin-induced ER stress in ATDC5 cells ATDC5 chondrosarcoma cells (RCB0565-Riken Cell Bank) were cultured as described (Altah et al. 2006). ER stress was induced by a 24 h treatment with thapsigargin (THG, Sigma) diluted in DMSO at the following concentrations: 0, 40, 80 and 160nM (Hamamura et al. 2009). All experiments were performed in triplicate and repeated twice.

Integrin blocking and recombinant CCN2 assays Sternal chondrocytes were isolated as described above. Integrin blocking assays were performed as previously described.
Briefly, sternal chondrocytes were plated and serum starved for 6–8 h. Chondrocytes were then treated for 24 h with an anti-rat integrin \( \alpha5 \) blocking antibody (1:100; CD49e BD Pharmingen) or 5 \% rat control serum (Sigma). In a separate set of experiments, ATDC5 cells were serum starved followed by a 1 h treatment with recombinant protein containing only the C terminal (CT) domain of CCN2 (150 ng/ml; Peprotech) or BSA added to the media, and then by a 1 h treatment with integrin \( \alpha5 \) blocking antibody and/or 80nM THG or DMSO control. Cells were lysed and RNA extracted using a Qiagen RNA purification kit following the manufacturer’s instructions. cDNA was generated using Superscript III (Invitrogen). The following primer sequences were used: Integrin alpha 5 Forward: 5’-AGGCATCTCTCACCATCTT-3’ and Reverse: 3’-TCAGGTTCACTGCGTTCTTG-5’, and normalized to GAPDH as described (Nishida et al. 2007).

**CCN2 overexpression in ATDC5 cells** ATDC5 cells were infected with a bicistronic adenovirus expressing CCN2 and green florescent protein (AdCCN2-GFP) adenovirus or an adenoviral control (Ad-CNT) vector for 24 h at a multiplicity of infection of 200 (University of Iowa; Hall-Glenn et al. 2012). After 24 h, cells were analyzed by immunofluorescence. Experiments were performed in triplicate and repeated twice.

**Western blot analysis** Proteins were isolated from Ccn2fxTg and WT sterna through lysis in RIPA buffer with 1X protease (Roche) and 1X phosphatase inhibitors (Sigma). 30 \( \mu \)g of protein lysates were separated by gel electrophoresis and transferred to 0.45 \( \mu \)m nitrocellulose membranes (Biorad). Membranes were blocked in milk and incubated at a 1:2,000 dilution of the following primary antibodies overnight at 4 \( ^\circ \)C: CCN2 (L-20; Santa Cruz Biotech) and \( \beta \)-actin (Sigma). The blots were incubated with the following secondary antibodies: Donkey anti-goat horseradish peroxidase (HRP) and Goat anti-rabbit HRP (1:5,000; Biorad). Membranes were developed using Pierce ECL HRP chemoluminescent reagent (ThermoScientific). The blots were repeated twice.

**Quantitative reverse transcriptase PCR (qRT-PCR)** All qRT-PCR reactions were performed with a SYBR Green Real-time PCR Master Mix (Fermentas) with a Mx3005P QPCR System (Stratagene). Relative expression of Ccn2, Bip and Chop were quantitated and normalized to Gapdh as described and performed in triplicate (Hamamura et al. 2009; Kawaki et al. 2008). Atg12 and Lc3 levels were quantitated and normalized to Gapdh as previously described (Kouroku et al. 2007; Marino et al. 2010). Statistical analysis

Immunofluorescent quantitation of the levels of CCN2, BiP and CHOP expression was performed through ImageJ analysis and calculated as a percentage of DAPI positive total cell counts. Three images were taken per independent experiment, followed by quantitation and averaging. At least three independent WT and mutant littermate growth plates were examined with each marker. All in vitro experiments were performed in triplicate and repeated twice. All graphs are represented as fold induction over normalized untreated controls. A normal distribution of the data was assumed, and statistical analysis was performed using Student’s \( t \)-test (95 \% confidence interval).

**Results**

Loss of CCN2 results in cellular stress in the growth plate

Ccn2 mutant growth plates and cultured chondrocytes exhibit decreased ECM production (Ivkovic et al. 2003; Nishida et al. 2007). However, the consequences on the overall organization of the cartilage ECM were not previously investigated. Therefore, transmission electron microscopy was performed on E18.5 WT and Ccn2 mutant growth plates. Unexpectedly, ultrastructural examination revealed enlarged and distended ERs in proliferating and hypertrophic chondrocytes in Ccn2 mutants (Fig. 1a–d). WT proliferating (Fig. 1a) and hypertrophic (Fig. 1c) chondrocytes contained an organized rough ER (rER) with a limited amount of protein evenly distributed throughout the cisternae. However, in Ccn2 mutants, rER cisternae were dilated (Fig. 1b, d). Large vacuoles filled with an electron-lucid granular substance were also observed in mutants, indicative of accumulated intracellular proteins (Fig. 1b, d). Moreover, the nuclear chromatin in mutant chondrocytes was condensed (Fig. 1d), indicating most chondrocytes were undergoing cell death. The mechanism by which chondrocytes undergo physiological cell death is a matter of debate, but condensed chromatin and TUNEL labeling are reliable markers of chondrocyte death (Ahmed et al. 2007). TUNEL staining showed that Ccn2\(^{−/−}\) growth plates exhibited an increase in the percent of chondrocytes undergoing cell death in both the proliferative and hypertrophic zones compared to WT growth plates (Fig. 1e–g). Ultrastructural analysis also revealed major defects in ECM assembly in mutants. Fewer collagen fibrils were observed throughout the growth plate in Ccn2 mutants compared to WT littermates (Fig. S1).

Because we noted distended ER in proliferative as well as hypertrophic chondrocytes, we examined CCN2 expression and confirmed that CCN2 protein is expressed both in the
proliferative and hypertrophic zones (Ivkovic et al. 2003) (Fig. 2a), and that CCN2 protein is absent in mutant growth plates (Fig. 2b) (Ivkovic et al. 2003). Next, we tested whether the distended ER in 
\textit{Ccn2} mutant chondrocytes is associated with activation of ER stress pathways by examining expression of the UPR activator, BiP, and the apoptosis inducing protein, CHOP. Consistent with previous studies (Tsang et al. 2007), low levels of BiP were observed in the hypertrophic zone (Fig. 2c), and CHOP (Fig. 2c) was undetectable in E16.5 WT growth plates. Increased expression of BiP (Fig. 2d) and CHOP (Fig. 2f) was observed in the hypertrophic zones of 
\textit{Ccn2} mutant growth plates, but not in the proliferative zone. Expression of calnexin, a calcium dependent ER chaperone, was elevated throughout the proliferative and prehypertrophic zones in 
\textit{Ccn2} mutants (Fig. 2g, h). Although calnexin is upregulated by ER stress in chondrocytes (Vranka et al. 2001), the observation that BiP and CHOP were not elevated in the proliferative zone in 
\textit{Ccn2} mutants indicates that these chondrocytes are not undergoing a classical UPR. Nonetheless, these results show that the absence of CCN2 in the growth plate results in defective ECM assembly, increased cellular stress, and chondrocyte cell death in both the proliferative and hypertrophic zones.

CCN2 Is a stress responsive gene in vitro

The finding that loss of CCN2 was associated with increased chondrocyte stress raised the possibility that CCN2 may be responsive to cellular stress in chondrocytes. Therefore, we tested whether 
\textit{Ccn2} is upregulated in chondrocytes during chemically induced ER stress. Thapsigargin (THG) induces cellular stress by inhibiting sarco/endoplasmic reticulum calcium ATPases, thus blocking calcium release from ER stores (Yang et al. 2005). Prior to cell death, the transcription of ECM components is downregulated and protective ER stress response genes such as BiP are activated by THG in chondrocytes (Yang et al. 2005). ATDC5 chondrocytic cells were treated with increasing concentrations of THG. BiP and Chop levels were examined using qRT-PCR. Consistent with previous studies, THG induced dose-dependent increases in BiP (Fig. 3a) and Chop mRNA (Fig. 3b) (Hamamura et al. 2009). In contrast to the expression of ECM components, which are
downregulated by THG (Nugent et al. 2011), Ccn2 mRNA levels were increased by THG with the same kinetics as Bip and Chop (Fig. 3c), suggesting that CCN2 is a stress-responsive gene in chondrocytes in vitro.

CCN2 depletion induces cellular stress in chondron cultures

The above experiments demonstrate that CCN2 expression is induced by stress in vitro. However, they do not demonstrate that the cellular stress observed in Ccn2−/− growth plates is due to a direct role for CCN2 in chondrocytes. Recent studies have shown that the presence of ECM protects chondrocytes from ER stress (Nugent et al. 2011). Therefore, a 3-dimensional culture system was developed in which chondrocytes were maintained in alginate, yielding chondrocyte clusters (chondrons) (Fig. S2) (Lee et al. 1997). The accumulation of ECM components can be observed (Fig. S2A, B). THG treatment induced cellular stress in chondrons, with Bip localization transitioning from the ER lumen in control cells (Fig. S2C), to the nucleus/perinuclear region in THG-treated chondrons (Fig. S2D). The re-localization of Bip to the nucleus has not been reported previously in chondrocytes, but is consistent with previous reports in other cell types (Ni et al. 2011). Chop mRNA levels were also increased in THG-treated chondrons (Fig. S2F), compared to untreated controls (Fig. S2E). Therefore, chondrons in alginate are sensitive to THG-induced stress. Next, the impact of loss of Ccn2 on THG-induced stress was tested. First, we confirmed that CCN2 is expressed in WT chondrons (Fig. 4a) and is absent in Ccn2−/− chondrons (Fig. 4b, c). WT chondrons expressed low levels of Bip (Fig. 4d). Ccn2−/− chondrons displayed upregulated Bip expression and a higher proportion of nuclear Bip-positive cells (Fig. 4e, f). CCN2 mutant chondrons also exhibit increased expression of Chop (Fig. 4g–i). These results suggest that CCN2 plays a direct role in the protection of chondrocytes against THG-induced stress in vitro.

The protective effect of CCN2 against chondrocyte stress may be mediated through integrin α5

CCN2 is thought to mediate its functions by acting as a ligand for integrins (Jun and Lau 2011). CCN2 induces expression of integrin α5, and binds to integrin α5β1 to promote chondrocyte proliferation and ECM secretion (Nishida et al. 2007). Integrin α5β1 is also required for chondrocyte survival (Pulai et al. 2002). Recent data demonstrate that multiple stress pathways are activated as a survival mechanism during cell detachment from the ECM (Avivar-Valderas et al. 2011). Taken together, these findings suggest that one of the prosurvival functions of integrin α5β1 in chondrocytes may be the suppression of cellular stress. To test this possibility, sternal chondrocytes were treated with or without an integrin α5-blocking antibody for 24 h (Fig. 5a). Bip and Chop mRNA expression levels were upregulated in response to treatment with the integrin α5-blocking antibody (Fig. 5a). Consistent with our findings that loss of Ccn2 leads to increased cellular stress in chondron cultures (Fig. 4), recombinant C-terminal CCN2 (rCCN2)-treated ATDC5 cells exhibited a decrease in Chop mRNA levels compared to BSA treated controls in the presence of THG (Fig. 5b). The C-terminal domain of rCCN2 contains the integrin α5β1-binding domain (Gao and Brigstock 2006). Next, ATDC5 cells were pre-treated for 1 h with recombinant C-terminal rCCN2 added to the medium prior to stress induction for 1 h in the presence of the α5-blocking antibody. As in Fig. 5a, treatment with integrin α5-blocking antibody led to increased Chop mRNA expression (Fig. 5c, columns 1 and 2), whereas treatment of ATDC5 cells with THG leads to an approximately 2-fold induction in Chop mRNA (Fig. 5b, columns 1 and 2). Also, treatment with THG in the presence of the integrin α5-blocking antibody leads to an approximately 10-fold induction of Chop (Fig. 5c, columns 1 and 3). Moreover, while rCCN2 prevented THG-induced Chop expression (Fig. 5b, columns 2 and 4), treatment with integrin α5-blocking antibody prevented the protective effect of rCCN2 (Fig. 5c,
Fig. 4 CCN2 depleted chondrons exhibit elevated cellular stress. Primary sternal chondrocytes were isolated from E16.5 WT and Ccn2−/− sterna, and cultured in alginate for 10 days to yield chondrons. CCN2, BiP and CHOP protein expression were analyzed through immunofluorescence. a–c CCN2 expression. CCN2 protein expression was abundant in WT chondrons (a), where punctate CCN2 protein expression was observed intracellularly (arrowhead) and pericellularly (arrows). CCN2 protein was not detected in Ccn2 mutant chondrons. b, c Quantitation of number of CCN2-expressing cells. d–f BiP expression in WT and Ccn2−/− chondrons. d BiP is detected in the ER in some cells in WT chondron clusters. e Nearly all Ccn2−/− chondrocytes express BiP, and many nuclei are positive. f Quantitation of the percentage of nuclear BiP-expressing cells. g–i CHOP expression. g Few WT chondrocytes express CHOP. In contrast, most Ccn2−/− chondrocytes express CHOP (H). i Quantitation of the percentage of CHOP-expressing chondrocytes. c, f, i CCN2, BiP, and CHOP expression was analyzed in triplicate using ImageJ analysis and calculated as a percentage of DAPI positive total cell counts. Asterisks indicate statistical significance with a p-value of 0.003. Open triangles in (c), (f), and (i) represent the averages of the data points. Representative images are shown.

Fig. 5 Blocking Integrin α5 results in chondrocyte stress. Primary sternal chondrocytes were serum starved and treated with anti-integrin α5 blocking antibody or goat serum (control) for 24 h, after which BiP and CHOP mRNA levels were quantitated by qRT-PCR and normalized to GAPDH. BiP and CHOP expression were increased by treatment with the blocking antibody. b rCCN2 protects against THG-induced stress, resulting in Chop mRNA induction. c This affect is attenuated upon treatment with α5 blocking antibody and THG compared to DMSO and rat serum controls.
columns 3 and 6). These results, although preliminary, suggest that integrin α5 may be required for the ability of CCN2 to protect against cellular stress in chondrocytes in vitro.

Overexpression of CCN2 attenuates chondrocyte stress

Since loss of CCN2 induces cellular stress in cartilage, we generated transgenic mice that overexpress CCN2 in chondrocytes to determine whether increased CCN2 could lead to reduced stress. An inducible transgenic construct was generated, consisting of a floxed transcriptional STOP signal placed upstream of the Ccn2 coding sequence (Fig 6a). Transgenic lines were established and mated to Col2Cre mice (Ovchinnikov et al. 2000) to induce chondrocyte-specific overexpression of CCN2 (Fig 6a). A 3–5 fold induction of CCN2 protein was observed in Ccnfx2Tg;Col2Cre mice (Fig. 6b). The specificity of induction was confirmed in isolated mouse embryonic fibroblasts (MEFs) from Ccnfx2Tg embryos infected with adenoviral Cre (Ad-CRE) (Fig. 6c). Ccnfx2Tg;Col2Cre mice were viable with no obvious morphological differences at birth (Fig. 6d), but exhibited progressive overgrowth of cartilage elements (data not shown). A complete characterization of these transgenics will be presented elsewhere. The growth plates of P0 Ccnfx2Tg tibiae had normal chondrocyte organization, although both the hypertrophic zone and chondrocytes within it were slightly smaller than in WT littermates (Fig. 6e, f). Consistent with a protective function for CCN2, levels of BiP were reduced in Ccnfx2Tg;Col2Cre mice compared to WT controls (Fig. 6g, h).

CCN2 regulates NFκB expression and autophagy

NFκB is required for chondrocyte survival (Park et al. 2007; Wu et al. 2007). CCN2 induces nuclear localization of NFκB and activates pro-survival mechanisms, while inhibiting stress-mediated apoptosis in a number of cell types (Gao and Brigstock 2005). NFκB is activated by integrin α5β1 in breast cancer epithelial cells (Sen et al. 2010) and α5β1 is a major receptor for CCN2 in chondrocytes (Sen et al. 2010). Our earlier results suggested that CCN2 protects against chondrocyte stress through
integrin α5 in vitro (Fig. 5). We therefore investigated whether CCN2 might exert pro-survival functions in chondrocytes at least in part through regulation of NFκB. NFκB localization was examined in E16.5 WT and mutant growth plates. In WT proliferative zones, NFκB was predominantly localized to the nucleus (Fig. 7a), similar to previous reports (Park et al. 2007; Wu et al. 2007). In Ccn2−/− growth plates, NFκB was present in the cytoplasm, but the number of cells exhibiting nuclear localization was decreased (Fig. 7b,c). These data are thus consistent with previous in vitro studies showing that CCN2 induces NFκB activation (Gao and Brigstock 2005), and raise the possibility that CCN2 exerts pro-survival effects in chondrocytes at least in part through NFκB.

Autophagy is an important survival mechanism in normal chondrocytes and in response to stress (Lotz and Carames 2011). We examined the expression of autophagosome markers Atg12 and LC3 (Atg8), which conjugate with one another to stabilize autophagosome formation (Mehrpour et al. 2010). In E16.5 WT growth plates, Atg12 and LC3 were expressed throughout the growth plate, with the highest levels in the hypertrophic zone (Fig. 7d, f), consistent with previous literature showing that autophagy is required for terminal chondrocyte differentiation (Srinivas et al. 2009). However, in Ccn2−/− growth plates Atg12 and LC3 protein (Fig. 7e,g) and mRNA (Fig. 7h, i) levels were decreased.

Because NFκB and autophagy have pro-survival functions in chondrocytes, we investigated whether CCN2 can induce NFκB and the expression of autophagy genes. ATDC5 cells infected with a CCN2 overexpressing adenovirus (Ad-CCN2-GFP) exhibited increased CCN2 expression within 24 h after infection (Fig. 8a, b). Consistent with in vivo studies (Fig. 7), CCN2 expression induced nuclear NFκB expression (Fig. 8c, d). Furthermore, CCN2 stimulated the expression of LC3 (Fig. 8e, f). These data suggest that CCN2 may protect against chondrocyte stress by upregulating pro-survival NFκB and autophagy pathways.

![Fig. 7 Decreased NFκB and autophagy-mediated cell survival in Ccn2 mutants.](image)

**A** In WT growth plates, NFκB was primarily localized to the nucleus throughout the proliferative zone. **B** In Ccn2−/− growth plates, NFκB expression in the proliferative zone was decreased and localized mainly to the cytoplasm. **C** NFκB nuclear expression throughout the growth plate was quantitated using Image J analysis as the percentage of DAPI positive nuclei. Growth plate immunostaining was performed in triplicate; *asterisk* denotes a p-value of 0.003, indicating statistical significance. Open triangle indicates the average of the data points. **D, E** E16.5 WT (D) and Ccn2−/− (E) growth plates immunostained for Atg12. **F, G** E16.5 WT (F) and Ccn2−/− (G) growth plates immunostained for Atg8 (LC3). All sections were counterstained with DAPI.

Stains were performed at least twice and representative images are shown. **H, I** Atg12 and LC3 mRNA levels were quantitated by qRT-PCR in WT and Ccn2−/− sternal chondrocytes.
Discussion

The data reported here provide evidence that CCN2 is important for ECM assembly and chondrocyte survival in the growth plate. Ultrastructural analysis revealed that loss of CCN2 leads to defective ECM organization and chondrocyte death, accompanied by increased cellular stress. The activation of stress pathways in vivo was confirmed by elevated levels of BiP and CHOP in Ccn2−/− growth plates. These results were recapitulated in vitro, where loss of Ccn2 in chondron cultures also resulted in cellular stress. This finding suggests that the role of CCN2 in protecting chondrocytes from stress is direct, rather than an indirect consequence of defective growth plate angiogenesis. The finding that Ccn2 expression is induced by stress, unlike structural ECM proteins, whose expression is downregulated, suggests that an important function of CCN2 may be to mediate chondrocyte survival.

The precise mechanisms by which CCN2 protects against cellular stress remain unclear. Integrin α5β1 is essential for chondrocyte survival in vitro (Pulai et al. 2002). Integrin α5 expression was decreased in Ccn2−/− chondrocytes, leading to defective chondrocyte adhesion and downstream integrin signaling (Nishida et al. 2007). Evidence that CCN2 may exert a pro-survival effect through modulation of cell-ECM attachment comes from the finding that treatment of chondrocytes with an integrin α5-blocking antibody leads to increased cellular stress, while treatment of ATDC5 cells with C-terminal rCCN2 (which contains the α5β1 binding site) (Gao and Brigstock 2006) mediates a modest protective effect during chemically induced stress, which is attenuated in the presence of an integrin α5-blocking antibody. Currently, no in vivo studies have been conducted to assess the role of α5 containing integrins in cartilage. However, the loss of β1 integrin leads to reduced ECM production, growth plate disorganization and impaired survival (Aszodi et al. 2003), consistent with a potential role for α5β1 in ECM organization and chondrocyte survival.

In addition to induction of pro-apoptotic stress pathways, the loss of Ccn2 also results in decreased expression of nuclear NFκB and autophagy genes in vivo. In accordance, overexpression of Ccn2 in chondrocytes resulted in induction of nuclear NFκB and autophagy gene expression. A limitation of these experiments is that the ability of overexpression of CCN2 to rescue NFκB and autophagy gene expression in Ccn2−/− chondrocytes was not tested in vitro. Thus, we cannot determine whether the effect of CCN2 is direct or indirect. However, both NFκB and autophagy exert pro-survival functions in growth plate chondrocytes (Srinivas et al. 2009; Wu et al. 2007). The mechanism by which CCN2 induces the expression of NFκB in chondrocytes is unknown but probably occurs through integrin signaling, as previously observed in other cell types (Gao and Brigstock 2005; Zahir et al. 2003).

CCN2 expression is correlated with the onset of osteoarthritis (OA) and synovial damage (Blaney Davidson et al. 2006; Omoto et al. 2004). Paradoxically, recombinant CCN2 expedites repair of articular cartilage during chemically induced knee OA (Nishida et al. 2004). These opposing effects of CCN2 may be due to its ability to modulate NFκB. While NFκB has pro-survival functions in chondrocytes (Park et al. 2007; Wu et al. 2007), it also promotes the expression of pro-inflammatory genes in OA chondrocytes (Marcu et al. 2010). Whether NFκB has a pro-survival function in OA chondrocytes is unknown. Further investigations of the mechanism by which CCN2 promotes...
survival in growth plate chondrocytes, and its role in the maintenance of articular cartilage may provide new avenues to treat and prevent OA.

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