CCN2 Is Necessary for Adhesive Responses to Transforming Growth Factor-β1 in Embryonic Fibroblasts*

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CCN2 is induced by transforming growth factor-β (TGFβ) in fibroblasts and is overexpressed in connective tissue disease. CCN2 has been proposed to be a downstream mediator of TGFβ action in fibroblasts; however, the role of CCN2 in regulating this process is unclear. By using embryonic fibroblasts isolated from ccn2−/− mice, we showed that CCN2 is required for a subset of responses to TGFβ. Affymetrix genome-wide expression profiling revealed that 942 transcripts were induced by TGFβ greater than 2-fold in ccn2−/+ fibroblasts, of which 345 were not induced in ccn2−/− fibroblasts, including pro-adhesive and matrix remodeling genes. Whereas TGFβ properly induced a generic Smad3-responsive promoter in ccn2−/− fibroblasts, TGFβ-induced activation of focal adhesion kinase (FAK) and Akt was reduced in ccn2−/− fibroblasts. Emphasizing the importance of FAK and Akt activation in CCN2-dependent transcriptional responses to TGFβ in fibroblasts, CCN2-dependent transcripts were not induced by TGFβ in faκ−/− fibroblasts and were reduced by wortmannin in wild-type fibroblasts. Akt1 overexpression in ccn2−/− fibroblasts rescued the TGFβ-induced transcription of CCN2-dependent mRNA. Finally, induction of TGFβ-induced fibroblast adhesion to fibronectin and type I collagen was significantly diminished in ccn2−/− fibroblasts. Thus in embryonic fibroblasts, CCN2 is a necessary cofactor required for TGFβ to activate the adhesive FAK/Akt/phosphatidylinositol 3-kinase cascade, FAK/Akt-dependent genes, and adhesion to matrix.

Growth factors intimately contribute to the normal wound healing process, regulating chemotaxis, cell proliferation, neovascularization, and extracellular matrix (ECM) synthesis. CCN2 (connective tissue growth factor), a member of the CCN family of proteins, contains 38 conserved cysteine-rich residues and a heparin-binding domain and is chemotactic and mitogenic for connective tissue cells (1–4). However, the physiological role of CCN2 is largely unknown.

As an initial approach to elucidate the physiological function of CCN2, mice deleted for the ccn2 gene were recently generated (5). Mice homozygous for a deletion of the ccn2 gene died soon after birth, displaying an inability of the rib cage to ossify properly (5). The phenotype of these mice is consistent with a role for CCN2 in matrix synthesis and remodeling as ccn2−/− embryos show reduction in the expression of bone-specific matrix proteins, such as aggrecan (5). We recently found that embryonic fibroblasts isolated from ccn2−/− mice showed reduced basal adhesive signaling, including a reduction of FAK and ERK phosphorylation and delays in α-smooth muscle actin (α-SMA) stress fiber formation (6), suggesting that CCN2 plays a key role in mediating the formation of attachments between the cell and matrix at focal adhesions.

Although CCN2 was discovered over a decade ago, the precise biological function of CCN2 has remained elusive. CCN2 is expressed in mesenchymal cells in development, is induced during wound healing (4, 8), and is overexpressed in fibrosis (7–11). Indeed, an enzyme-linked immunosorbent assay detecting the amino-terminal portion of the CCN2 protein has indicated that the appearance of CCN2 in the blister fluid of scleroderma patients can be considered an excellent surrogate marker for the severity of skin fibrosis (12). One of the most potent inducers of CCN2 is TGFβ, which promotes CCN2 expression in dermal fibroblasts, but not in epidermal keratinocytes, through a complex network of transcriptional interactions requiring Smads, protein kinase C, and Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase/ERK and a consensus transcription enhancer factor binding-element in the CCN2 promoter (11, 13). CCN2 protein production in response to TGFβ does not occur in smad3−/− mouse embryonic fibroblasts (MEFs) or in the presence of the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor U0126 (10, 14).

As CCN2 is induced by TGFβ, it has been hypothesized that CCN2 is a downstream mediator of TGFβ action (15). Indeed, this notion is the chief operating paradigm in the field. In fact, it is unclear whether virus; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; RT, real time; α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEAP, secreted enhanced alkaline phosphatase.

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3 The abbreviations used are: ECM, extracellular matrix; TGFβ, transforming growth factor-β; FAK, focal adhesion kinase; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; MEF, mouse embryonic fibroblast; PI, phosphatidylinositol; CMV, cytomegalovirus; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; RT, real time; α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEAP, secreted enhanced alkaline phosphatase.
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ionically CCN2 is a downstream mediator or co-activator of TGFβ action. Furthermore, the contribution of CCN2 to particular TGFβ signaling pathways is unknown. Finally, although CCN2 has been proposed to be required for TGFβ responses in fibroblasts, but not in epithelial cells (15), whether CCN2 is required for all, or a subset, of TGFβ responses in fibroblasts is completely unknown.

In this study, we take advantage of the fact that CCN2 is expressed by MEFs and is induced by TGFβ in this cell type (10). Consequently, examining the impact of loss of CCN2 expression by MEFs is likely to be a useful predictive tool to assess the impact of CCN2 in development, but should significantly affect our understanding of CCN2 action in pathological conditions where CCN2 is constitutively expressed, such as cancer and fibrosis (16). Such an approach should also allow an appreciation of the potential effect of anti-CCN2 therapies on these pathologies. Thus, in this study, we perform Affymetrix gene profiling, Western blot, and real time (RT)-PCR analyses to evaluate the contribution of CCN2 to the activity of TGFβ in MEFs. Our results provide new insights into the role of CCN2 in mesenchymal biology.

MATERIALS AND METHODS

Cell Culture and Harvesting—ccn2+/+ and ccn2−/− MEFs (embryonic day 14.5) were isolated as described previously (5) and grown in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), and 1 mM sodium pyruvate (Invitrogen). Cells were grown at 37 °C, 5% CO2, harvested to SDS-PAGE and pellets were collected after centrifugation at 2000 rpm for 5 min at 4 °C and resuspended in 2% SDS, quantified (BCA kit; Pierce), and placed in Laemmli sample buffer containing complete protease and phosphates inhibitors mixture (Roche Applied Science). fak+/+ and fak−/− MEFs (American Type Culture Collection) were similarly cultured.

Western Blot Analysis—Equal amounts of protein (20 µg) were subjected to SDS-PAGE. Gels were electrophoretically transferred to nitrocellulose (Invitrogen). Membrane was blocked with 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween 20 (Sigma), and immunoblotting was performed using anti-phospho-ERK, anti-ERK, anti-FAK, or anti-phospho-FAK antibodies (Cell Signaling Technology), anti-CCN2 (Abcam), anti-type I collagen (BIODESIGN International), anti-α-SMA, and anti-vinculin (Sigma) antibodies as described by the manufacturer. Anti-GAPDH and anti-vimentin (Sigma) antibodies were used as loading controls. Blots were then developed by incubation with biotinylated anti-rabbit or anti-mouse antibodies (1:1000; Vector Laboratories) as secondary antibodies, followed by incubation with ABC reagent (Vector Laboratories). Signal was detected using a luminescence kit (ECL kit; Amersham Biosciences) and x-ray film. Densitometry was performed using Gel Base/Gel-Blot Pro (Synoptics).

Cell Transfections—Transfections of ccn2+/+ and ccn2−/− MEFs were performed essentially as described previously (11, 13). Briefly, 2 × 105 cells were seeded into each well of a 6-well plate. The next day, cells were transfected using FuGENE (Roche Applied Science) in a ratio of 3 µl of FuGENE per 2 µg of DNA (1.5 mg of reporter, 0.5 µg of CMV-β-galactosidase (Clontech)). Luciferase expression was determined and adjusted for β-galactosidase expression, which was used to control for differences in transfection efficiencies among wells (Applied Biosystems). Experiments were performed three times in triplicate. A representative experiment is shown. In rescue experiments, cells were similarly transfected with either a vector encoding constitutively active Akt1 or an empty expression vector (Upstate Biotechnology). Cells were incubated after transfection for 24 h in serum-free media, followed by further incubation in the presence or absence of 4 ng/ml TGFβ1 for 6 h. RNA was harvested and subjected to real time-PCR analysis. To verify the functionality of the Akt1 construct, a reporter DNA construct containing multimers of an NFκB-responsive element upstream of the secreted enhanced alkaline phosphatase gene (NFκB-SEAP; Clontech) was transfected into NIH 3T3 fibroblasts (American Type Culture Collection) using PolyFect (Qiagen) at a ratio of 10 µl of PolyFect per 2 µg of DNA (0.25 mg of CMV-β-galactosidase; 0.25 µg of NFκB-SEAP; 1 µg of empty expression vector or expression vector containing constitutively active Akt1).

RNA Quality Assessment, Probe Preparation, and GeneChip Hybridization and Analysis—Microarrays and analysis were performed essentially as described previously (17, 18). All GeneChips were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada). RNA was harvested (Trizol, Invitrogen) and quantified, and quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) and the RNA 6000 Nano kit (Caliper Life Sciences). Quality data were then analyzed using the degraderomat (mean degradation factor 1.99, S.D. 0.0678). Biotinylated cRNA was prepared from 10 µg of total RNA as per the Affymetrix GeneChip Technical Analysis Manual (Affymetrix). Double-stranded cDNA was synthesized using SuperScript II (Invitrogen) and oligo(dT)24 primers. Biotin-labeled cRNA was prepared by cDNA in vitro transcription using the Bizarre High Yield RNA Transcript Labeling kit (Enzo Biochem) incorporating biotinylated UTP and CTP. Fifteen µg of labeled cRNA was hybridized to Mouse Genome 430 2.0 GeneChips for 16 h at 45 °C as described in the Affymetrix Technical Analysis Manual (Affymetrix). GeneChips were stained with streptavidin/phycocerythrin, followed by an antibody solution, and a second streptavidin/phycocerythrin solution, with all liquid handling performed by a GeneChip Fluidics Station 450. GeneChips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Signal intensities for genes were generated using GCOS1.2 (Affymetrix) using default values for the statistical expression algorithm parameters and a target signal of 150 for all probe sets and a normalization value of 1. Normalization was performed in GeneSpring 7.2 (Agilent Technologies Inc.). The Robust Multichip Average preprocessor was used to import data from the .cel files. Data were first transformed (measurements less than 0.01 set to 0.01) and then normalized per chip to the 50th percentile and per gene to wild-type control samples. 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 to identify a transcript as being altered. These criteria had to be met in both sets of experiments. Clustering using gene ontologies was filtered using a 2-fold cutoff using the level two filtering parameter by the Affymetrix website. Probes annotated by the Gene Ontology Consortium were used. Categories that were considered over-represented in the set of transcripts that were CCN2-dependent had to have at least four members.

Real Time-PCR—Cells were serum-starved for 24 h and treated with 4 ng of TGFβ for different lengths of time, as indicated. Total RNA was isolated using Trizol (Invitrogen), and the integrity of the RNA was verified by gel electrophoresis or Agilent Bioanalyzer. For initial time course analysis, total RNA (25 ng) was reverse-transcribed and amplified using TaqMan Assays on Demand (Applied Biosystems) in a 15-μl reaction volume containing two unlabeled primers and 6-carboxyfluorescein-labeled TaqMan MGB probe. Samples were combined with TaqMan one-step mastermix (Applied Biosystems). Amplified sequences were detected using the ABI Prism 7900 HT sequence detector (PerkinElmer Life Sciences) according to the manufacturer’s instructions. Triplicate samples were run; transcripts were measured in picograms, and expression values were standardized to values obtained with control 28 S RNA primers as described previously (19). Statistical analysis was performed by the Student’s paired t test.

Adhesion Assay—Adhesion assays were performed essentially as described (6). 96-Well plates were coated overnight at 4 °C with 4 μg/ml type I collagen, 4 μg/ml fibronectin, or 4 μg/ml bovine serum albumin (Sigma) in 1× PBS. Nonspecific binding sites on substrates were blocked for 1 h in 1% BSA in PBS at room temperature prior to addition of cells. Fibroblasts were cultured in DMEM, 0.5% fetal calf serum for 24 h and then were harvested with 2 mM EDTA in PBS (20 min, room temperature), washed twice with DMEM containing 0.5% BSA (Sigma), and resuspended in the same medium at 2.5 × 10^5 cells/ml. Cells (100 μl/well) were added to wells of the 96-well plate and incubated at 37 °C for 1 h.

FIGURE 2. RT-PCR time course analysis of the induction by TGFβ1 of mRNAs in ccn2+/+ and ccn2−/− MEFs. CCN2 is a co-factor for the TGFβ1 induction of a subset of mRNAs. MEFs isolated from ccn2+/+and ccn2−/− mice dermal fibroblasts were cultured in DMEM, 0.5% FBS for 24 h and treated for TGFβ1 (4 ng/ml) for 2, 6, and 24 h. RT-PCR was used to detect CCN2, αSMA, COL1A1, COL1A2, and MMP-3 mRNA. Induction of α-SMA, COL1A1, and COL1A2 depended on CCN2, as addition of TGFβ to ccn2−/− MEFs impaired the induction of these mRNAs at all time points tested, paralleling the induction of CCN2 mRNA. Note that TGFβ1 induction of MMP-3 mRNA occurred in ccn2−/− MEFs.

FIGURE 3. The Smad signaling pathway is functional in ccn2−/− MEFs. A generic Smad-responsive promoter (SBE-lux, a luciferase reporter gene driven by multiple copies of a Smad3-responsive element) was transfected into ccn2+/+ and ccn2−/− MEFs and treated with TGFβ1 (4 ng/ml) for 24 h. Expression of the reporter gene luciferase was calculated, relative to control β-galactosidase expression from a co-transfected control CMV-β-galactosidase vector. Average values ± S.D. are shown. * indicates significant induction of promoter activity by TGFβ1; p < 0.05.
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TABLE 1

Transcripts induced by TGFβ greater than 2-fold in Ccn2+/+ and Ccn2−/− MEFs

| Affymetrix ID | Fold change WT | Fold change KO | GenBank™ accession no. | Description |
|---------------|----------------|----------------|------------------------|-------------|
| Matrix production, contraction, cell adhesion | | | | |
| 1419149_at | 11 | 10 | NM_008871 | Serine (or cysteine) proteinase inhibitor, clade E, member 1 |
| 1449660_s_at | 7 | 7 | AW548837 | Coronin, actin-binding protein 1C |
| 1421315_s_at | 6 | 4 | B688355 | Cortactin |
| 1423541_at | 6 | 7 | BB377873 | Chondroitin sulfate proteoglycan 4 |
| 1466911_at | 5 | 5 | AW744319 | Procollagen, type V, α1 |
| 1417500_at | 5 | 4 | RC016492 | Transglutaminase 2, C polypeptide |
| 1416687_at | 5 | 3 | BC013152 | Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2 |
| 1450379_at | 4 | 4 | NM_010833 | Moesin |
| 1419089_at | 4 | 4 | BI11620 | Tissue inhibitor of metalloproteinase 3 |
| 1423407_at | 4 | 3 | BF228318 | Fibulin 2 |
| 1460302_at | 4 | 4 | A185532 | Thrombospondin 1 |
| 1417534_at | 4 | 3 | NM_010580 | Integrin b5 |
| 146157_at | 4 | 3 | NM_009502 | Vinculin |
| 1450944_at | 3 | 3 | BB377873 | Chondroitin sulfate proteoglycan 4 |
| 1419648_at | 3 | 3 | NM_008659 | Myosin IC |
| 1418726_a_at | 3 | 5 | NM_011619 | Troponin T2, cardiac |
| 1448228_at | 3 | 3 | ME6143 | Lysyl oxidase |
| 1452469_at | 3 | 3 | BFS78669 | Smoothelin |
| 1452783_at | 3 | 3 | AK005500 | Fibronectin type III domain containing 3B |
| 1426750_at | 3 | 3 | AW538200 | Filamin, β |
| 1452545_a_at | 3 | 3 | U37029 | Integrin β1 |
| 1451097_at | 3 | 2 | CO15289 | Vasodilator-stimulated phosphoprotein |
| 1427385_at | 2 | 2 | BC003232 | Actinin, α1 |
| 1425896_a_at | 2 | 2 | AF007248 | Fibrillin 1 |
| 1448546_at | 2 | 2 | NM_007687 | Cofilin 1, non-muscle |
| 1449335_at | 2 | 2 | BI11620 | Tissue inhibitor of metalloproteinase 3 |
| 1417917_at | 2 | 2 | NM_009922 | Calponin 1 |

Cell signaling

| Affymetrix ID | Fold change WT | Fold change KO | GenBank™ accession no. | Description |
|---------------|----------------|----------------|------------------------|-------------|
| 1421207_at | 8 | 7 | AF065917 | Leukemia inhibitory factor |
| 1427682_a_at | 7 | 11 | X06746 | Early growth response 2 |
| 1417409_at | 5 | 5 | NM_010591 | juv oncogene |
| 1415899_at | 4 | 5 | NM_008416 | juv-B oncogene |
| 1418572_x_at | 4 | 5 | NM_013749 | Tumor necrosis factor receptor superfamily, member 12a |
| 1448998_at | 4 | 5 | NM_008350 | Interleukin 11 |
| 1448593_at | 4 | 5 | NM_018865 | WNT1-inducible signaling pathway protein 1 |
| 1422885_at | 4 | 3 | NM_009921 | Runt-related transcription factor 1 |
| 1422414_a_at | 4 | 3 | NM_007589 | Calmodulin 2 |
| 1422485_at | 3 | 3 | AK004804 | SMAD 4 |
| 1418901_at | 3 | 3 | NM_009883 | CCAAT/enhancer binding protein (C/EBP), β |
| 1438123_at | 3 | 3 | BM202770 | Cysteine-rich protein 61 |
| 1448605_at | 3 | 4 | NM_007484 | Ras homolog gene family, member C |
| 1448594_at | 3 | 4 | NM_018865 | WNT1-inducible signaling pathway protein 1 |
| 1429946_at | 3 | 2 | B687997 | Smurfl |
| 1417148_at | 3 | 3 | NM_008809 | Platelet-derived growth factor receptor, β polypeptide |
| 1421534_at | 3 | 3 | NM_008016 | Fibroblast growth factor-inducible 15 |
| 1448694_at | 3 | 3 | NM_010591 | juv oncogene |
| 1423389_at | 3 | 3 | BF221666 | SMAD 7 |
| 1452163_at | 2 | 2 | BI151715 | Ets1 |
| 1428395_at | 2 | 3 | B8832916 | Smurf1 |
| 1448304_at | 5 | 5 | NM_024287 | RAB6, member RAS oncogene family |

Cell metabolism

| Affymetrix ID | Fold change WT | Fold change KO | GenBank™ accession no. | Description |
|---------------|----------------|----------------|------------------------|-------------|
| 1438160_x_at | 18 | 7 | AV348121 | Solute carrier organic anion transporter family, member 4a1 |
| 141757_at | 7 | 7 | NM_010162 | Exotoxins (multiple) |
| 1417262_at | 7 | 8 | M94967 | Prostaglandin-endoperoxide synthase 2 |
| 1429776_a_at | 7 | 7 | AK005680 | DnaJ (Hsp40) homolog, subfamily B, member 6 |
| 1426645_at | 6 | 4 | AU079047 | Heat shock protein 1, α |
| 1450886_at | 5 | 5 | BB729616 | Nuclear protein 5 |
| 1422459_at | 5 | 5 | NM_011875 | Proteasome 26 S subunit, non-ATPase, 13 |
| 1426790_at | 4 | 4 | BC024835 | Structure specific recognition protein 1 |
| 1439041_at | 3 | 2 | BM239325 | Solute carrier family 22 (zinc transporter), member 10 |
| 1417462_at | 4 | 3 | NM_007598 | CAP, adenylyl cyclase-associated protein 1 |

for 60 min. An acid phosphatase assay was used, in which adherent cells were quantified by incubation with 100 μl of substrate solution (0.1 M sodium acetate, pH 5.5, 10 mM p-nitrophenyl phosphate, and 0.1% Triton X-100) for 2 h at 37 °C. The reaction was stopped by the addition of 15 μl of 1 N NaOH/well, and A405 was measured. Statistical analysis was performed by the Student’s unpaired t test.

RESULTS

CCN2 Induced α-SMA and Type I Collagen but Not Induced MMP-3 or Smad3-responsive Promoter—CCN2 protein is induced by TGFβ yet is expressed at a basal level in MEFs (9) (Fig. 1). To assess whether CCN2 was required for transcriptional responses to TGFβ, real-time-PCR was performed on mRNAs prepared at various time points after TGFβ treatment of cccn2+/+ and cccn2−/− MEFs. Primers detecting matrix metalloproteinase-3 (MMP-3), COL1A1, COL1A2, and α-SMA were selected for these initial analyses, given that these are known markers of an activated fibroblast (20).

To our surprise, the ability of TGFβ to induce COL1A1, COL1A2, and α-SMA was impaired even at the earliest time points, paralleling the induction of CCN2 mRNA in cccn2+/+ fibroblasts (Fig. 2). Results were con-
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TABLE 2

| Affymetrix ID | Fold change WT | Fold change KO | GenBank™ accession no. | Description |
|---------------|----------------|---------------|------------------------|-------------|
| Matrix production, contraction, cell adhesion |
| 1443534_at | 5 | 1.5 | BM206272 | Filamin, β |
| 1430533_at | 4 | 1.5 | B1134907 | Catenin β |
| 1448383_at | 3 | 1.5 | NM_008608 | Matrix metalloproteinase 14 |
| 1426519_at | 3 | 1.5 | A314028 | Proline 4-hydroxylase, α1 |
| 1416573_at | 3 | 1 | NM_008608 | Matrix metalloproteinase 14 |
| 1418230_at | 3 | 1 | RC005621 | LIM and senescent cell antigen-like domains 1 |
| 1445965_at | 3 | 1 | BR037433 | RIKEN cDNA C920271 gene |
| 1431834_at | 2 | 1 | AK020015 | Elastin microfilibrer interphase 1 (Emilin1) |
| 1456632_at | 2 | 1.5 | BM232388 | Tropomyosin 1, α |
| 1438556_a_at | 2 | 1 | BR224629 | Tropomodulin 3 |
| 1418440_at | 2 | 1 | NM_007739 | Procollagen, type VIII, α1 |

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| 1434413_at | 3 | 0.5 | BG092677 | Insulin-like growth factor 1 |
| 1436740_at | 3 | 1 | B1692255 | Protein kinase, lysine-deficient 1 |
| 1420899_at | 3 | 1.5 | AW542340 | RAB18, member RAS oncogene family |
| 1425711_a_at | 3 | 1 | M94335 | Thymoma viral proto-oncoprotein 1 |
| 1436585_at | 3 | 1 | BB003847 | Muscle blind-like 2 |
| 1418186_at | 3 | 1 | AF146523 | Receptor (calcinium) activity modifying protein 2 |
| 1419156_at | 2 | 1 | AI628101 | SRY-box containing gene 4 |
| 1437383_at | 2 | 1 | BB173900 | Latent transforming growth factor β-binding protein 3 |
| 1443832_s_at | 2 | 1 | AV064339 | Serum deprivation response |
| 1418532_at | 2 | 1 | BB371106 | Frizzled homolog 2 (Drosophila) |
| 1434962_at | 2 | 1 | NM_029744 | Phosphatidylcholine 4-kine type 2 β |
| 1445355_at | 2 | 1 | BM223600 | G protein-coupled receptor 21 |
| 1417092_at | 2 | 1 | BC013446 | Parathyroid hormone receptor 1 |
| 1459984_at | 2 | 1 | A1552688 | Melanoma inhibitory activity 3 |

Cell metabolism |
| 1450933_at | 3 | 1 | BG070255 | Phosphodiesterase 7A |
| 1423531_s_at | 3 | 1 | BG070255 | Phosphodiesterase 7A |
| 1454249_s_at | 2 | 1 | BF236176 | ATP-binding cassette, subfamily F (GCN20), member 1 |

firmed using Western blot analyses employing anti-α-SMA and anti-type I collagen antibodies (Fig. 1). Conversely, TGFβ was able to induce MMP-3 in the absence of CCN2 (Fig. 2). These results suggest that the ability of CCN2 to mediate TGFβ-induced gene expression is dependent on the basal, constitutive expression of CCN2 by MEFs rather than on the TGFβ-induced increment of CCN2 expression. Given that MMP-3 was induced by TGFβ in ccn2+/− MEFs, these results suggest that CCN2 is not generally required for TGFβ-dependent transcriptional responses but, in the context of MEFs, that CCN2 may be a cofactor for TGFβ action.

To confirm the notion that CCN2 was not generally required for the ability of TGFβ to induce gene expression, ccn2+/+ and ccn2−/− fibroblasts were transfected with a DNA construct bearing multiple copies of a Smad-responsive element subcloned upstream of the luciferase reporter gene (SBE-lux; Fig. 3). This element is responsive to activation of the Smad3 pathway, which is required for TGFβ responses in fibroblasts (21). The generic TGFβ-responsive Smad signaling pathway was fully active in ccn2−/− MEFs as the ability of the Smad reporter to respond to TGFβ was not impaired in ccn2−/− MEFs (Fig. 3). Collectively, these results suggest that CCN2 was not generally required for TGFβ signaling in fibroblasts but instead affects a specific subset of TGFβ-dependent responses.

Affymetrix Gene Profiling of Con2+/+ and Ccn2−/− Fibroblasts Reveal Novel Genes Whose Induction by TGFβ Is CCN2-dependent—To evaluate to what extent CCN2 was required for the ability of TGFβ to induce gene expression by MEFs, we cultured ccn2+/+ and ccn2−/− MEFs until 80% confluence and serum-starved cells for 24 h. Cells were then treated in the presence or absence of TGFβ (4 ng/ml) for an additional 6 h. This time point was chosen because our real time-PCR analysis indicated that TGFβ responses in MEFs were maximal at 6 h. Total RNA was prepared from these cells, reverse-transcribed, and applied to Affymetrix MOE430 arrays. Experiments were performed twice, and average induction values were obtained. Analysis of data by GeneSpring revealed TGFβ induced 942 transcripts greater than 2-fold in ccn2+/+ fibroblasts. Of these 345 were not induced in ccn2−/− fibroblasts. A representative selection of transcripts is shown in Tables 1 and 2. That the majority of TGFβ-induced genes in ccn2+/+ were also induced in ccn2−/− MEFs supported our initial impression that CCN2 was not generally required for the ability of TGFβ to induce gene expression. Pro-fibrotic (adhesion, contraction, and matrix) genes, as revealed by cluster analysis, were revealed to be both independent and dependent on CCN2 (Tables 1 and 2). Similarly, genes involved with cell signaling and metabolism were both independent and dependent on CCN2 (Tables 1 and 2). No significant physiological grouping specific to ccn2-dependent or ccn2-independent genes emerged. However, analysis of functional clusters indicated that genes significantly over-represented in the group nonresponsive to TGFβ in ccn2−/− fibroblasts were those involved with RNA processing and transcription. A complete list of these mRNAs is shown in Table 3. These results indicated that loss of CCN2 expression in MEFs is likely to both directly and indirectly affect gene expression in response to TGFβ (Tables 2 and 3).

To confirm the requirement for target gene induction by TGFβ for CCN2, we verified our gene array data using real time-PCR analysis of RNA isolated from ccn2+/+ and ccn2−/− MEFs treated with and without TGFβ for 6 h (Fig. 4). Whereas TGFβ induced mRNA encoding the matrix protein COL2A1 and the focal adhesion protein vinculin (22) in ccn2−/− MEFs, the ability of TGFβ to induce mRNAs encoding the matrix remodeling protein MMP14 (matrix metalloproteinase 14) (23), the collagen processing enzyme prolyl 4-hydroxylase (24), the cytoskeletal gene TPM1 (tropomyosin 1) (25), and the matrix and pro-adhesive proteins LIMS1 (26), Akt1 (27), and Emilin1 (28) were significantly impaired in the absence of CCN2 expression.

TGFβ-induced Akt/PI 3-Kinase and FAK Are Impaired by Loss of CCN2—We then wished to gain insights into the signaling pathways responding to TGFβ that were deficient in ccn2−/− MEFs. After our...
initial experiments ruled out the Smad pathway, we reasoned that loss of ccn2−/− must affect the ability of TGFβ to signal through other routes. Loss of CCN2 expression resulted in a reduced ability for TGFβ to induce LIMS1, Akt1, and Emilin1 mRNA, proteins involved with adhesive signaling. CCN2 binds adhesive receptors including integrins and heparan sulfate-containing proteoglycans (6, 29–31). In addition, ccn2−/− MEFs showed reduced adhesion to and signaling on fibronectin (6). Thus, we reasoned that loss of CCN2 might affect the ability fibroblasts to respond to TGFβ by inducing adhesive signaling. To confirm this notion, we harvested protein extracts prepared from ccn2−/− MEFs treated with and without 6 h showed that the ccn2−/− dependent genes prolyl 4-hydroxylase, COL1A1, and α-SMA by TGFβ also depended on FAK expression (Fig. 6). Conversely, the ccn2−/− independent gene vinculin was induced by TGFβ in a fashion independent of FAK (Fig. 6).

In fibroblasts, PI 3-kinase acts within the integrin/FAK/Akt cascade (32). Confirming the notion that impaired FAK/Akt signaling in response to TGFβ was responsible for the inability of ccn2−/− MEFs to respond to TGFβ, we performed real time-PCR analysis of ccn2−/− MEFs treated with and without TGFβ for 6 h showed that the ccn2−/− dependent genes prolyl 4-hydroxylase, COL1A1, and α-SMA by TGFβ also depended on FAK expression (Fig. 6). Conversely, the ccn2−/− independent gene vinculin was induced by TGFβ in a fashion independent of FAK (Fig. 6).
these results are consistent with the notion that in embryonic fibroblasts CCN2 is required for the ability of TGFβ to signal through Akt/PI3-kinase.

To extend our analysis, we wished to assess whether overexpressing constitutively active Akt1 in ccn2−/− fibroblasts could now allow the expression of CCN2-dependent transcripts in response to TGFβ.

FIGURE 4. Identification of novel CCN2-dependent targets; a subset of the transcriptional responses to TGFβ in MEFs is significantly impaired in ccn2−/−MEFs. MEFs isolated from ccn2+/+ and ccn2−/− mice dermal fibroblasts were cultured in DMEM, 0.5% FBS for 24 h and treated for TGFβ1 (4 ng/ml) for 6 h. RT-PCR was used to detect matrix metalloproteinase-14 (MMP-14), tropomyosin-1 (TPM-1), vinculin, Akt1, prolyl 4-hydroxylase (P4H), LIMS1, COL2A1, and Emilin1. Induction of MMP14, TPM1, Akt1, prolyl 4-hydroxylase, LIMS1, and Emilin1 were impaired ccn2−/− MEFS. Conversely induction of COL2A1 and vinculin occurred in ccn2−/− MEFS. * indicates significant induction of mRNA by TGFβ; p < 0.05. WT, ccn2+/+; WTT, ccn2+/++ TGFβ; KO, ccn2−/−; KOT, ccn2−/− + TGFβ.

verify the functionality of our construct encoding constitutively active Akt1, we showed that co-transfection into fibroblasts of an expression vector constitutively active Akt1, but not empty expression vector, could activate an Akt1-responsive promoter. To perform this analysis, we used an NFκB-responsive promoter (NFκB-SEAP), as NFκB is activated by Akt in fibroblasts (33). As anticipated, co-transfection of an
expression vector constitutively active Akt1, but not empty expression vector, resulted in a significant activation of SEAP reporter gene expression, verifying functionality of the construct encoding constitutively active Akt1 (Fig. 9A). Extending these results, and confirming our Western blot data showing reduced basal Akt activity in ccn2−/− MEFs (Fig. 5), we showed that, when NFκB-SEAP (which is non-TGFβ-responsive; data not shown) was transfected into ccn2+/+ and ccn2−/− fibroblasts, reporter activity was significantly impaired in ccn2−/− fibroblasts, yet was rescued with Akt1 overexpression (Fig. 9A), giving support to the notion that Akt-dependent transcriptional responses are impaired in the absence of CCN2.

We then investigated whether the construct encoding constitutively active Akt1, as compared with empty expression vector, could rescue the ability of TGFβ to induce expression of target mRNAs in ccn2−/− MEFs. We verified, using real time-PCR analysis of RNAs isolated from transfected MEFs, that we were able to achieve Akt1 overexpression in ccn2−/− MEFs transfected with expression vector encoding Akt1 (Fig. 9B). As earlier data examining the effect of loss of CCN2 expression on the induction of a Smad3-responsive promoter suggested that MEFs transfected poorly, we focused our rescue experiments on the mRNA expression of prolyl 4-hydroxylase and α-SMA, because the pattern of expression of these mRNAs suggested a high response to TGFβ in ccn2+/+ cells, and thus examination of these mRNAs would enhance our chances of observing a statistically significant alteration in transcriptional responses to Akt1 in transfected ccn2−/− cells. Unfortu-
Akt1 at least partially restored the ability of MEFs of type I collagen mRNA precluded the use of this transcript for this experiment. We found that transfection of constitutively active Akt1 at least partially restored the ability of ccn2−/− cells to respond to TGFβ by inducing prolyl 4-hydroxylase and α-SMA (Fig. 9C, P4H and α-SMA). Conversely, overexpression of Akt1 seemed to partially attenuate the ability of ccn2−/− cells to respond to TGFβ by inducing vinculin mRNA (Fig. 9C, vinculin). These latter results presumably reflect that overexpression of active Akt1 results in a bias in TGFβ responsiveness toward Akt1-dependent genes and away from Akt1-independent genes. Collectively, our results point to a critical role of CCN2 in mediating the TGFβ induction of adhesion and adhesive signaling in MEFs.

**CCN2 Is Required for TGFβ-induced Adhesion**—Based on our analysis of the effect of loss of CCN2 on gene expression and signaling in response to TGFβ, and our previous data showing that a principal defect of ccn2−/− fibroblasts was a decreased ability of cells to adhere to fibronectin (6), we reasoned that loss of CCN2 might affect the ability of MEFs to adhere to extracellular matrix proteins after TGFβ treatment. To evaluate this hypothesis and provide a functional context for our microarray and signal transduction analyses, we treated ccn2+/+ and ccn2−/− MEFs with TGFβ for 24 h, and cell adhesion to fibronectin and type I collagen was assessed. As anticipated, we found that loss of CCN2 resulted in reduced cell adhesion to matrix, consistent with our previous data showing that CCN2 is required for maximal cell adhesion to fibronectin (6). However, although TGFβ was able to enhance the ability of ccn2+/+ MEFs to adhere to fibronectin and type I collagen, ccn2−/− MEFs were unable to respond to TGFβ by an increase in adhesion to ECM (Fig. 10). These results provide functional support to our array and signaling data and suggest that adhesive signaling in response to TGFβ is impaired in ccn2−/− MEFs compared with ccn2+/+ MEFs. Collectively, our results support the notion that a principal, physiological role of CCN2 is to modulate adhesive responses in fibroblasts to ECM and growth factors.

**DISCUSSION**

TGFβ is a potent profibrotic cytokine; yet, as TGFβ possesses multiple functions, antagonizing TGFβ in chronic fibrotic diseases is likely to have substantial side effects (34, 35). Consequently, identification of a specific modulator of TGFβ is likely to be of significant impact in the development of selective therapies for disease. Although it has long been hypothesized that CCN2 is a downstream mediator of TGFβ action (15), the mechanism of action of CCN2 is largely unknown. Functional data indicate that overexpression of CCN2 itself may have little effect (36, 37) but may require a co-stimulus for its activity (36, 37). CCN2 directly binds to TGFβ and appears to increase binding to TGFβ to the TGFβ receptors and activation of a Smad-responsive promoter in Xenopus cells at extremely low TGFβ concentrations (38); however, the functional relevance of these observations is unknown. Overall, whether CCN2 modulates particular TGFβ signaling pathways and the physiological effect of loss of CCN2 to mammalian cells are unclear.

In this study, for the first time we show that CCN2 is a selective mediator of TGFβ action in MEFs. TGFβ is able to induce a majority of TGFβ-responsive genes in ccn2−/− MEFs and activate a generic Smad3-responsive promoter; however, a significant minority of TGFβ-responsive genes depends on CCN2. TGFβ induction of type I collagen and α-SMA is impaired by loss of CCN2 expression, even at very early time points. TGFβ-induced FAK and Akt phosphorylation is impaired in ccn2−/− MEFs. ccn2−/− MEFs showed defects in adhesive signaling in response to TGFβ and whether these responses were relevant to
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FIGURE 9. The lack of TGFβ induction of CCN2-dependent transcripts in ccn2−/− MEFs is alleviated by overexpression of constitutively active Akt1. A, functionality of construct encoding constitutively active Akt1 and reduction of Akt-dependent reporter (NFκB-SEAP) expression in ccn2−/− MEFs. An Akt1-responsive promoter (NFκB-SEAP) was transfected into ccn2+/+ and ccn2−/− MEFs in the presence or absence of empty expression vector (EMPTY) or expression vector encoding constitutively active Akt1 (Akt1). SEAP expression was monitored 48 h later, as described under “Materials and Methods.” A significant reduction in NFκB-dependent reporter gene expression in ccn2−/− MEFs was observed. Relative to the empty expression vector, co-transfection of expression vector encoding active Akt1 resulted in an induction of NFκB-dependent reporter activity in both ccn2+/+ and ccn2−/− MEFs (n = 3; p < 0.05). B, transfaction of expression vector encoding Akt1 results in expression of Akt1 mRNA. MEFs isolated from ccn2−/− mice fibroblasts were transfected with empty expression vector (CONTROL) or constitutively active Akt1 (Akt1) and treated for TGFβ1 (4 ng/ml) for 6 h. RT-PCR was performed with primer sets detecting Akt1. Transfection of expression vector encoding Akt1, under control of the non-TGFβ-responsive viral cytomegalovirus promoter, results in the overexpression of Akt1 mRNA. C, demonstration that transfection of expression vector encoding Akt1 at least partially rescues the TGFβ induction of CCN2-dependent transcripts in ccn2−/− MEFs. mRNAs isolated in B were subjected to real-time PCR analysis with primers detecting prolyl 4-hydroxylase (P4H) and α-SMA (* indicates significant induction of mRNA by TGFβ; p < 0.05). Conversely, Akt1 overexpression reduced the induction of vinculin mRNA (#, p < 0.05), reflecting that overexpression of Akt1 results in a bias of TGFβ-induced mRNA expression in favor of Akt1-dependent transcripts and away from Akt-1-independent transcripts.

FIGURE 10. Enhanced adhesion MEFs to fibronectin and type I collagen in response to TGFβ is CCN2-dependent. MEFs isolated from ccn2+/+ and ccn2−/− mice dermal fibroblasts were cultured in DMEM, 0.5% FBS for 24 h, treated with TGFβ1 (4 ng/ml) for 24 h, detached with EDTA, and allowed to adhere to BSA, type I collagen, or fibronectin (4 μg/ml each) that had been coated on a 96-well plate. After a blocking step, fibroblasts were allowed to adhere for 40 min, and cell adhesion was assessed as described under “Materials and Methods” (6 wells/data point; average ± S.E. (error bar) are shown). The values obtained from the BSA control experiment, not significantly above background, indicate the very few cells nonspecifically adhering to the plate.

the induction of CCN2-dependent mRNA induction. Indeed, TGFβ induction of FAK and Akt phosphorylation was severely impaired in ccn2−/− MEFs, and the Akt/PI 3-kinase pathway was necessary for the induction of ccn2-dependent genes in ccn2+/+ MEFs in response to TGFβ. These results suggest that, in the context of “activated” fibroblasts such as MEFs that endogenously express CCN2 (10), CCN2 is a co-factor required for TGFβ induction of gene expression. A similar function of CCN2 would be expected in activated fibroblasts in vivo, e.g., in fibrotic cells (16) or in tumor stroma (39).

It is interesting to note that cluster analysis did not reveal categories of genes that were completely dependent on CCN2. It should be noted, however, that consistent with previous observations examining α-SMA stress fiber formation post-adhesion (6), CCN2 was not required for TGFβ-induced collagen gel contraction (data not shown). Intriguingly, we found that loss of CCN2 did not affect basal mRNA or protein expression of α-SMA or type I collagen, suggesting that CCN2 does not directly mediate the expression of these genes. These results emphasize the selective role of CCN2 as a mediator of TGFβ action, and are consistent with the notion that induction of mRNAs encoding several procontractile proteins were not affected by loss of CCN2, including vinculin. However, there were genes over-represented in those transcripts affected by the loss of CCN2, namely those involved with transcription and translation. Although beyond the scope of our current study, these results suggest that CCN2 may indirectly affect the expression of downstream target genes, in addition to being required as a cofactor to modulate TGFβ signaling. It is interesting to note that several of the mRNAs whose expression depended on CCN2, as revealed by array analysis, included the forkheads, which are Akt-responsive (40). In addition, it is likely that activation of downstream transcription factors that are Akt-responsive is impaired in ccn2−/− MEFs; indeed, we have shown that activation of a reporter gene driven by multiple copies of a binding
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Our results are consistent with the notion that CCN proteins in general, including CCN2, interact with different proteins and ligands involved in signaling and consequently act to bring together regulatory circuits and consequently modify their activity (3, 4).

In conclusion, although CCN2 was discovered over a decade ago, the physiological roles of CCN2 (and the signaling cascades through which CCN2 acts) are largely unknown. Our results, using MEFs, showing that CCN2 is required for the ability of TGFβ to induce FAK and Akt phosphorylation and for adhesion of ECM, indicate a crucial role for CCN2 in mediating adhesive signaling in response to TGFβ. These results suggest that CCN2 plays a critical role in facilitating tissue remodeling and emphasize the specific role that CCN2 may play in situations where CCN2 is constitutively expressed, such as development, cancer, and fibrosis.

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