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Growth Differentiation Factor-15 (GDF-15) Suppresses In Vitro Angiogenesis Through a Novel Interaction With Connective Tissue Growth Factor (CCN2)

Ramon J. Whitson, Marshall Scott Lucia, and James R. Lambert*

Department of Pathology, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado 80045

ABSTRACT

Growth differentiation factor-15 (GDF-15) and the CCN family member, connective tissue growth factor (CCN2), are associated with cardiac disease, inflammation, and cancer. The precise role and signaling mechanism for these factors in normal and diseased tissues remains elusive. Here we demonstrate an interaction between GDF-15 and CCN2 using yeast two-hybrid assays and have mapped the domain of interaction to the von Willebrand factor type C domain of CCN2. Biochemical pull down assays using secreted GDF-15 and His-tagged CCN2 produced in PC-3 prostate cancer cells confirmed a direct interaction between these proteins. To investigate the functional consequences of this interaction, in vitro angiogenesis assays were performed. We demonstrate that GDF-15 blocks CCN2-mediated tube formation in human umbilical vein endothelial (HUVEC) cells. To examine the molecular mechanism whereby GDF-15 inhibits CCN2-mediated angiogenesis, activation of $\alpha_V\beta_3$ integrins and focal adhesion kinase (FAK) was examined. CCN2-mediated FAK activation was inhibited by GDF-15 and was accompanied by a decrease in $\alpha_V\beta_3$ integrin clustering in HUVEC cells. These results demonstrate, for the first time, a novel signaling pathway for GDF-15 through interaction with the matricellular signaling molecule CCN2. Furthermore, antagonism of CCN2 mediated angiogenesis by GDF-15 may provide insight into the functional role of GDF-15 in disease states. J. Cell. Biochem. 114: 1424–1433, 2013.

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KEY WORDS: GROWTH DIFFERENTIATION FACTOR-15 (GDF-15); MACROPHAGE INHIBITORY CYTOKINE-1 (MIC-1); PROSTATE DERIVED FACTOR (PDF); TRANSFORMING GROWTH FACTOR BETA (TGFβ); CONNECTIVE TISSUE GROWTH FACTOR (CTGF); CYR61, CTGF, AND NOV PROTEIN FAMILY 2 (CCN2); ANGIOGENESIS; FOCAL ADHESION KINASE (FAK)

Growth differentiation factor-15 (GDF-15) is a divergent member of the transforming growth factor beta (TGFβ)/bone morphogenetic protein (BMP) superfamily sharing only 15–29% sequence similarity with other members of this family [Bootcov et al., 1997]. GDF-15 is secreted as a mature dimer and is grouped with other members of the TGFβ family due to the conserved cysteine knot motif found within the mature peptide. GDF-15 has been identified in a variety of experimental systems and is also known as macrophage inhibitory cytokine-1 (MIC-1), prostate derived factor (PDF), non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1), placental bone morphogenetic protein (PLAB), and placental TGFβ (PTGFβ) [Bootcov et al., 1997; Hromas et al., 1997; Paralkar et al., 1998; Bottner et al., 1999; Li et al., 2000; Baek et al., 2001].

GDF-15 is primarily secreted from epithelial cells and is aberrantly expressed in tumors [Thomas et al., 2001; Karan et al., 2003; Bauskin et al., 2005; Lind et al., 2009]. Similar to TGFβ1, GDF-15 is reported to have both anti- and pro-tumorigenic properties [Baek et al., 2001; Lambert et al., 2006; Martinez et al., 2006; Cekanova et al., 2009; Senapati et al., 2010; Wang et al., 2011]. GDF-15 suppresses cell proliferation in breast, colon, and prostate cancer cells [Li et al., 2000; Baek et al., 2001; Lambert et al., 2006] and inhibits tumor growth in prostate xenografts [Lambert et al., 2006]. However, increased serum levels of GDF-15 are associated with metastatic disease in prostate cancer patients [Selander et al., 2007]. Despite the association between GDF-15 and cancer, a signaling pathway activated downstream of GDF-15 in tumors has not been established. As a member of the TGFβ family, we hypothesized that

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Ramon J. Whitson's present address is Department of Dermatology, Stanford University, Stanford, CA 94305.

*Correspondence to: Dr. James R. Lambert, Department of Pathology, University of Colorado Denver, Anschutz Medical Campus, 12801 E. 17th Avenue, M.S. 8401, Aurora, CO 80045. E-mail: jim.lambert@ucdenver.edu

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GDF-15 may activate classical TGFβ signaling through phosphorylation of the downstream effectors of TGFβ, Smad proteins. However, phosphorylation of Smad proteins (Smads 2, 3 and Smads 1, 5, 8) was not observed following GDF-15 treatment in a variety of cell types (unpublished data). In an effort to identify a potential alternate signaling pathway for GDF-15, interacting proteins were identified in a yeast two-hybrid screen. The pro-angiogenic protein, connective tissue growth factor (CTGF/CCN2), was identified as a GDF-15 binding partner.

Angiogenesis is a vital component for nutrient uptake and removal of waste products during development, wound repair, and in growing tumors [Folkman, 1995; Hanahan and Weinberg, 2000]. GDF-15 is reported to be a mediator of *N*-(4-hydroxyphenyl) retinamide antiangiogeneic activity [Ferrari et al., 2005] suggesting a role for GDF-15 in angiogenesis; however, the mechanism for this process remains poorly understood.

The CCN protein family is comprised of stromal derived factors which are misregulated during tumor development and cardiovascular disease [Chen and Lau, 2009; Dhar and Ray, 2010; Zuo et al., 2010]. CCN factors are named for the first three members of this family, cysteine rich-61 (Cyr61; CCN1), connective tissue growth factor (CTGF; CCN2), and nephroblastoma overexpressed (Nov; CCN3). Most CCN factors contain four conserved functional domains with homology to insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C (VWC), thrombospondin-1 (TSP1) and cysteine knot (CT) domains (Fig. 1A). CCN factors bind to components of the extracellular matrix (ECM) and interact with cell surface proteins, and thus, have been termed matri-cellular adapter proteins [Brigstock, 2003; Perbal, 2004].

CCN family members carry out pro-angiogenic biological functions during development, wound repair, and cancer [Babic et al., 1998; Chen et al., 2001; Brigstock, 2002, 2003; Lin et al., 2003; Perbal, 2004; Yang et al., 2005]. CCN2 is a direct regulator of endothelial cell activity [Lau and Lam, 1999; Shimo et al., 2001b; Brigstock, 2002; Kondo et al., 2002; Chien et al., 2011] and may play a role in tumor angiogenesis [Shimo et al., 2001a; Yang et al., 2005; Chu et al., 2008]. Indeed, vascular formation and tumor growth was enhanced in xenografts with CCN2 overexpression [Yang et al., 2005].

Here, we report interaction between GDF-15 and the VWC domain of CCN2 and that GDF-15 inhibits CCN2-mediated angiogenesis in vitro. GDF-15 suppressed CCN2-mediated activation of focal adhesion kinase (FAK) suggesting a novel anti-migratory mechanism for reduced endothelial cell activity by GDF-15.

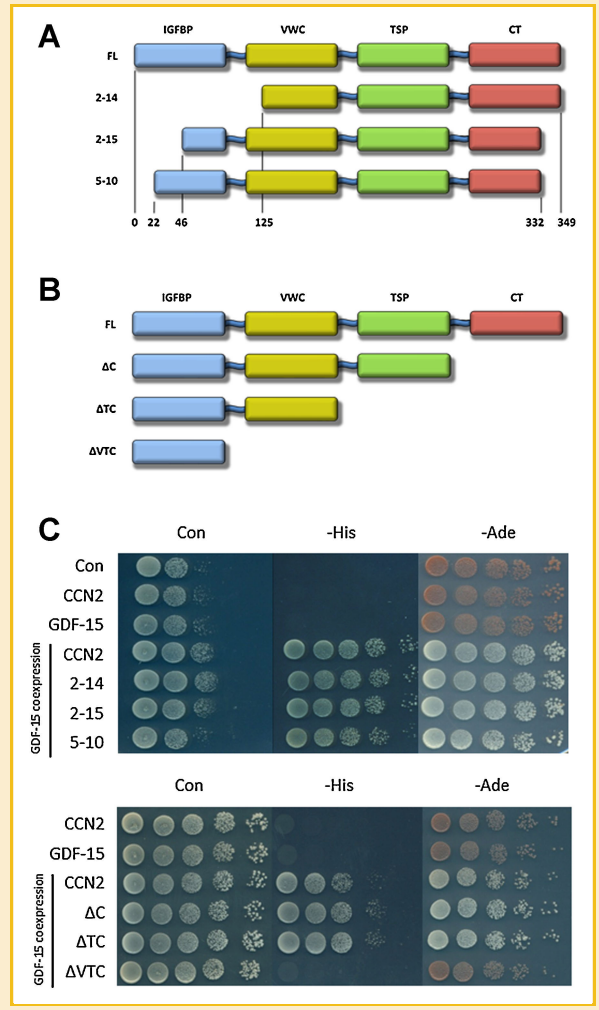

Fig. 1. Yeast two-hybrid screening identifies CCN2 as a binding partner of GDF-15. A: Schematic representation of the domain structure of full length (FL) CCN2 and cDNA library clones identified (2–14, 2–15, and 5–10) in the yeast two-hybrid screen. Numbers indicate amino acid residues. B: Full-length CCN2 and CCN2 domain deletion mutants used for domain mapping studies. ΔC, ΔTC, and ΔVTC represent deletion mutants of the CT, CT + TSP, and CT + TSP + VWC domains, respectively. C: Yeast growth assay detecting interactions in the yeast strain PJ69-4a co-transformed with the indicated Gal4 fusions. CCN2 constructs were in pACT2 and GDF-15 was expressed off pBD-Gal4. Serial dilutions of the transformants were plated on leucine/tryptophan dropout (Con), histidine dropout (-His), and adenine dropout (-Ade) media.

MATERIALS AND METHODS

CELL LINES AND CULTURE

PC3 human prostate cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial (HUVEC) cells (ATCC) were maintained in endothelial growth medium (C1556; Sigma–Aldrich, St. Louis, MO) supplemented with 10% FBS and penicillin–streptomycin. HUVEC cells were maintained on dishes coated with fibronectin (10 μg/ml).

ANTIBODIES

Polyclonal antiserum directed against GDF-15 was generated by Zymed as described previously [Lambert et al., 2006]. Affinity-purified anti-GDF-15 antibodies were prepared using the Pierce SulfoLink Coupling Resin according to the manufacturer's instructions. Rabbit polyclonal antibodies against CCN2 (L20; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-FAK (3284; Cell Signaling Technology), and mouse monoclonal antibodies against total FAK

(610087, BD Biosciences, Franklin Lakes, NJ) and β-actin (A5441, Sigma) were used for immunoblotting. Blocking antibody against GDF-15 was purchased from R&D Systems (BAF940; Minneapolis, MN). Mouse monoclonal antibody against integrin $\alpha_V\beta_3$ (MAB1976; Millipore, Billerica, MA) and 594-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) were used for immunofluorescence.

YEAST TWO-HYBRID SCREEN AND INTERACTION DOMAIN MAPPING

Two-hybrid interaction assays were carried out using the PJ69-4a yeast strain (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4(Δ) gal80(Δ) LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ). Yeast were maintained as described previously [James et al., 1996]. Two-hybrid screening was carried out using the Matchmaker cDNA library from U2-OS osteosarcoma cells (Clontech, Mountain View, CA). cDNA was expressed in the pACT2 expression vector. The DNA binding domain of Gal4 was fused to the mature peptide of GDF-15 in the pBD-Gal4 expression plasmid. All GDF-15 cDNAs were cloned into pBDGal4 using primers indicated in Supplementary Table III. All GDF-15 constructs were cloned into the *Eco*R1 and *Xho*1 restriction sites in pBD-Gal4 and sequences were confirmed by DNA sequencing.

Full length CCN2 and CCN2 domain deletion mutants (Fig. 1B) were cloned into pACT2 using primers indicated in Supplementary Table III. CCN2 constructs were cloned into the *Eco*R1 and *Xho*1 restriction sites of pACT2 and sequences were confirmed by DNA sequencing.

Yeast transformation was carried out according to manufacturer's directions (Clontech). Transformants were selected on SD-Trp, Leu plates. To identify proteins that interacted with GDF-15, colonies were screened for expression of Ade2, His3, and beta-galactosidase. Interactions were verified and quantified by measuring beta-galactosidase (β-Gal) activity using a liquid β-Gal assay kit (Thermo Scientific, Rockford, IL).

IMMUNOBLOT ANALYSIS

Cells were harvested by washing two times in 4°C PBS. Total cellular extracts were prepared by cell lysis in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) containing protease inhibitors (Protease Inhibitor Cocktail, Cat# P8340, Sigma–Aldrich) and phosphotase inhibitors (10 mM NaVo4, and 50 mM NaF). Protein concentration of each extract was determined by dye-binding assay (Bio-Rad, Hercules, CA). Samples were separated on 4–12% MES NuPAGE gels (Invitrogen) and transferred to PVDF membrane. Immunoblots were imaged using the Odyssey fluorescence labeling system (Li-COR Biosciences, Lincoln, NE).

GDF-15:CCN2 BIOCHEMICAL INTERACTION

Full length CCN2 containing a 7X Histidine tag (CCN2-His) was created using primers indicated in Supplementary Table III. CCN2-His was cloned into the *Eco*R1 and *Bam*H1 restriction sites in the multiple cloning region of pCDNA3.1. pCDNA3.1-CCN2 constructs were transfected into PC3 cells using Mirus transfection reagent (Mirus Bio LLC, Madison, WI). Cells were plated at 2.5×10^5 cells per well in a 6-well dish. Cells were transfected 24 h after plating and media was harvested 24 h after transfection. Media was diluted 1:10 in TBS containing 5% BSA and imidazole (50 mM). One milliliter of media/BSA solution was incubated with 40 μl of 50% slurry Ni-NTA resin (Qiagen, Germantown, MD) for 1 h at 4°C. Ni-NTA beads were washed 3× with PBS containing imidazole (50 mM) at 4°C. Beads were resuspended in 100 μl of elution buffer containing 250 mM imidazole, 100 mM DTT in PBS, and 25 μL NuPAGE loading dye (Life Technologies, Carlsbad, CA). Elution buffer/bead mix was incubated at 100°C for 10 min and 35 μl was loaded onto SDS PAGE gel for immunoblot analysis.

PREPARATION OF CONDITIONED MEDIUM

We used PC3 human prostate cancer cells as a biological source of GDF-15 because one of the primary tissue types that most highly express GDF-15 is the prostate. However, PC3 cells do not express GDF-15 and therefore serves as an appropriate source of control and GDF-15-containing conditioned medium following transfection of GDF-15 expression constructs. Conditioned medium was prepared from PC3 cells stably expressing GDF-15 (LXIN-GDF-15) or LXIN-Empty vector control (LXIN-Em). 1×10^6 cells were plated into a 10 cm dish containing 15 ml RPMI media supplemented with 2.5% FBS. Media was collected and cleared by centrifugation after 48 h incubation at 37°C. For CCN2 conditioned media, PC3 LXIN-Em cells were transfected with CCN2 expression vector (pCDNA3.1-CCN2) or empty vector control (pCDNA3.1-Em) using Mirus transfection reagent (Mirus Bio LLC). DNA transfection complex was created using 10 μg plasmid DNA diluted into 1 ml of OptiMEM medium (Life Technologies). Thirty microliters of Mirus reagent was added and mixture was incubated for 30 min. Complex was added to a 10 cm plate containing 1×10^6 PC3 LXIN-Em cells in 5 ml RPMI medium supplemented with 2.5% FBS. After 37°C incubation for 4 h, 10 ml of RPMI medium containing 2.5% FBS was added. Media were changed after 16 h at 37°C and replaced with 15 ml RPMI supplemented with 2.5% FBS. Cells were incubated for 48 h at 37°C and media collected. Media were frozen at −80°C for storage.

ENDOTHELIAL TUBE FORMATION

Tube formation assay using HUVEC cells was carried out in 96-well plates using the Cultrex endothelial tube formation kit (Trevegen, Helgerman, CT). A 1:1 mixture containing Cultrex basement membrane extract (BME) and reduced growth factor Matrigel BME (BD Biosciences) was prepared for a final volume of 40 μL per well. Ninety-six-well plates were pre-cooled at 4°C overnight. Subsequently, BME was placed into pre-cooled plates and incubated for 30 min at 37°C. HUVEC cells were suspended in serum free endothelial cell culture media (Sigma) and plated onto gelled BME at 2×10^4 cells in 50 μl of media per well. Condition media was generated as described above. Conditioned media was mixed with HUVEC cells for a final volume of 150 μl of media per well. Media volume was normalized using media from PC3-LXIN-Em cells transfected with empty vector control plasmid. GDF-15 blocking antibody and non-specific IgG incubated in conditioned media for 30 min prior to plating (both at 2 μg/ml final concentration). HUVEC tube formation was carried out for 2.5 h at which time each well was washed 2× in 200 μl cold PBS then placed in 200 μl 10% buffered

formalin. Five representative fields per condition were captured at 100× magnification. Cell junctions within each frame were counted using the multi-selection counter within ImageJ software (National Institutes of Health, Bethesda, MD). Cell counts from five fields were averaged and statistical testing carried out using Excel software (Microsoft, Redmond, WA).

IMMUNOFLUORESCENT STAINING AND CONFOCAL MICROSCOPY

Glass coverslips were coated with Matrigel (diluted 1:5 in endothelial media) and dried overnight in 35 mm dishes at room temperature. 1×10^5 HUVEC cells were plated per 35 mm dish. Cells were treated with 2 ml conditioned media (described above) for 1 h. After treatment, coverslips were submerged in 2 ml 10% buffered formalin for 20 min. Coverslips were washed two times with PBS then incubated with 2% horse serum for 30 min at room temperature. Coverslips were incubated with mouse anti-integrin $\alpha_V\beta_3$ antibody (10 μg/ml) diluted in 3% horse serum overnight at room temperature. Coverslips were washed two times with PBS and treated with goat anti-mouse secondary labeled with Alexa 488 or 594 (Molecular Probes, Eugene, OR) and diluted at 1:600 in 2% horse serum containing 5 μg/ml DAPI for 1 h at room temperature. Coverslips were washed two times with PBS and mounted onto glass slides. After drying for 30 min, cells were imaged at 600×. Immunofluorescent images were captured on a Nikon Diaphot fluorescence microscope equipped with a Cooke SensiCam CCD camera (Tonawand, NY) using Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO) as previously described [Russell et al., 2007]. Fluorescence images were digitally deconvolved using the No Neighbors algorithm (Slidebook) and converted to TIFF files. All images were processed by Photoshop (Adobe Systems, Inc., Mountain View, CA).

PHOSPHORYLATION OF FAK IN HUVEC CELLS

Six-well dishes were coated with 100 μl of matrigel (BD Biosciences) diluted fivefold in endothelial cell media (Sigma) and left to dry overnight. One milliliter of CCN2, GDF-15, control conditioned media or combination of indicated media was placed into each well. Volume of media was normalized in each well by adding 1 ml of control media to wells containing only CCN2 or GDF-15 resulting in a total of 2 ml for all wells. HUVEC cells were plated into each well at 2.5×10^5 cells per well. Cells were harvested 1.5 h after plating and cells extracts were prepared for immunoblot analysis as described above.

RESULTS

GDF-15 INTERACTS WITH CCN2

We and others have reported antitumorigenic activity for GDF-15 [Baek et al., 2001, 2006; Lambert et al., 2006; Martinez et al., 2006; Cekanova et al., 2009]; however, the signaling mechanism(s) whereby GDF-15 exerts its antitumorigenic properties remains unclear. To gain insight into the functional role of GDF-15, identification of proteins that interact with GDF-15 was pursued. A yeast two-hybrid screen using GDF-15 as bait against prey from a cDNA library generated from U2-OS osteosarcoma cells was carried out. To identify potential interacting proteins, and to eliminate false positives, several phenotypic assays activated by two-hybrid interaction in the PJ69-4a yeast strain were utilized. The phenotypic assays examined were growth on medium lacking histidine (SD-His), pigment change after growth on medium lacking adenine (SD-Ade), and induction of beta-galactosidase (β-Gal) visualized by x-gal metabolism. Using this screening methodology, an interaction between GDF-15 and three peptide fragments of connective tissue growth factor (CCN2) were identified (Fig. 1A,C). This interaction was validated by carrying out two-hybrid studies using GDF-15 and full length CCN2 (Fig. 1C).

TABLE I. β-Gal Activity in Yeast Coexpressing GDF-15 and Full-Length CCN2 (CCN2-FL) or CCN2 Clones From Two-Hybrid Screen

Plasmids	p-Gal activity	SEM	*P*-value
pBDGal4 + pACT2-CCN2-FL	2.48	±0.09	–
pBDGal4-GDF-15 + pACT2	2.99	±0.20	0.11
pBDGal4-GDF-15 + pACT2-CCN2-FL	39.89	±0.07	3.7×10^{-6}
pBDGal4-GDF-15 + pACT2-2-14	22.61	±0.84	1.2×10^{-3}
pBDGal4-GDF-15 + pACT2-2-15	18.32	±0.21	7.2×10^{-5}
pBDGal4-GDF-15 + pACT2-5-10	45.47	±3.40	6.2×10^{-3}

Negative controls consisted of pACT2-CCN2-FL or GDF-15 cotransformed with pBD-Gal4 and pACT2 empty vectors, respectively. *P* values were calculated from triplicate experiments using Students *t*-test for respective values compared to CCN2-FL expression alone.

CCN2 carries out distinct signaling activities through each of its functional domains [Perbal, 2004; Chen and Lau, 2009]. To examine CCN2 signaling activities that may be affected by GDF-15, the CCN2 domain that interacts with GDF-15 was determined (Fig. 1B,C). Two-hybrid binding studies were performed using GDF-15 and CCN2 constructs lacking specified domains (Fig. 1B). Growth on SD-His plates and pigment change on SD-Ade plates was observed for all deletion mutants except CCN2ΔVTC (Fig. 1C) suggesting that GDF-15 binds to the VWC domain of CCN2. A quantitative approach to measure relative binding activity between GDF-15 and full length CCN2 or CCN2 domain deletion mutants was utilized. β-Gal activity expressed after two-hybrid interaction was measured using a liquid β-Gal reporter assay. β-Gal activity was high after co-transformation with GDF-15 and CCN2-FL, or CCN2 clones 2–14, 2–15, and 5–10 from our two-hybrid screen (Table I). β-Gal was high for CCN2 domain deletion mutants ΔC, and ΔTC, but not ΔVTC (Table II), further indicating that GDF-15 binds to the VWC domain of CCN2.

A polymorphism at position 6 of the mature peptide of GDF-15 (H6D) is reported to affect prostate cancer outcome [Lindmark et al.,

TABLE II. β-Gal Activity in Yeast Coexpressing GDF-15 and Full-Length CCN2 (CCN2-FL) or CCN2 Domain Deletion Mutants

Plasmids	p-Gal activity	SEM	*P*-value
pBDGal4 + pACT2-CCN2-FL	1.68	±0.04	–
pBDGal4-GDF-15 + pACT2	2.05	±0.20	0.20
pBDGal4-GDF-15 + pACT2-CCN2-FL	21.86	±1.59	3.6×10^{-3}
pBDGal4-GDF-15 + pACT2-ΔC	42.35	±3.46	4.7×10^{-3}
pBDGal4-GDF-15 + pACT2-ΔTC	30.50	±1.63	1.8×10^{-3}
pBDGal4-GDF-15 + pACT2-ΔVTC	1.72	±0.10	0.24

Negative controls contain CCN2-FL or GDF-15 expressed with pBD-Gal4 and pACT2 empty vectors, respectively. *P* values calculated using Students *t*-test for respective values compared to CCN2-FL expression alone.

2004; Hayes et al., 2006]. To determine if this polymorphism affects binding to CCN2, β-Gal activity in yeast co-transformed with GDF-15-H6D and CCN2 was measured. β-Gal activity for GDF-15-H6D was similar to GDF-15-wild type (Supplementary Table I) suggesting that interaction between CCN2 and GDF-15 is not affected by the H6D polymorphism.

Similar to other members of the TGFβ family, GDF-15 is secreted as a mature dimer composed of peptides which are cleaved from a full-length form termed, pro-GDF-15. Aberrant stromal accumulation of pro-GDF-15 in the prostate is associated with increased risk of relapse in prostate cancer patients [Bauskin et al., 2005]. To determine if CCN2 interacts with pro-GDF-15, β-Gal activity was measured in yeast co-transformed with CCN2 and pro-GDF-15. Compared to mature GDF-15, β-Gal activity was decreased significantly in yeast transformed with pro-GDF-15 (Supplementary Table II) indicating that CCN2 does not interact with pro-GDF-15.

SECRETED GDF-15 INTERACTS WITH CCN2

In human cells, GDF-15 and CCN2 are secreted into the extracellular milieu [Bootcov et al., 1997; Bauskin et al., 2000]. To validate GDF-15:CCN2 interaction from our yeast studies, binding studies were carried out when these factors were secreted from human prostate cells. GDF-15 and His-tagged CCN2 were co-expressed and secreted into media after co-transfection of expression vectors into PC3 prostate cancer cells (Fig. 2). CCN2-His was pulled down using Ni-NTA agarose beads. Immunoblotting with anti-GDF-15 antibody indicated that GDF-15 was complexed with CCN2-His (Fig. 2). These data indicate that GDF-15 binds to CCN2 when secreted from PC3 cells providing biochemical validation of our yeast two-hybrid results for GDF-15:CCN2 interaction.

GDF-15 INHIBITS CCN2-MEDIATED ANGIOGENESIS IN VITRO

CCN2 is a direct regulator of endothelial cell activity [Lau and Lam, 1999; Shimo et al., 2001b; Brigstock, 2002; Kondo et al., 2002; Chien et al., 2011]. Expression of CCN2 promotes pro-angiogenic activity in HUVEC cells [Kireeva et al., 1997; Babic et al., 1999; Shimo et al., 1999]. We reasoned that GDF-15:CCN2 interaction may affect CCN2-activated angiogenesis. An endothelial tube formation assay was carried out using HUVEC cells. Endothelial cells were treated with conditioned media containing GDF-15 and CCN2. Tube formation was increased in cells treated with CCN2 conditioned media; however, tube formation was inhibited after co-treatment with GDF-15 (Fig. 3A). This effect was blocked when HUVEC cells were treated with a blocking antibody against GDF-15 (Fig. 3A). In vitro angiogenesis was quantified by counting cell junctions formed during tube formation (Fig. 3B), as described previously [Arnaoutova et al., 2009; Arnaoutova and Kleinman, 2010]. Formation of cell junctions is increased with CCN2 treatment which is blocked after co-treatment with GDF-15 (Fig. 3B). Treatment with a blocking antibody against GDF-15 resulted in recovery of CCN2-mediated cell junction formation. To test if CCN2-activated angiogenesis is blocked non-specifically by IgG treatment, HUVEC cells were treated with a non-targeting control antibody. CCN2-activated cell junction formation was not affected by non-specific antibody control (Supplementary Fig. 1). These data suggest that GDF-15 inhibits the pro-angiogenic activity of CCN2 in vitro.

Angiogenic activity of endothelial cells is promoted by many factors including FGF-2 [Chabut et al., 2003]. To determine if GDF-15 alters FGF-2-activated angiogenesis, HUVEC cells were co-treated with GDF-15 and FGF-2 and formation of cell junctions was measured. GDF-15 did not block FGF-2-mediated tube formation (Supplementary Fig. 2). This suggests that suppression of angiogenesis by GDF-15 is unique to CCN2-mediated angiogenesis in the experiments presented.

The VWC domain of CCN family member Cyr61 is required for endothelial cell activation [Chen et al., 2004]. Among CCN family members CCN2 shares the strongest sequence similarity to Cyr61. To determine the requirement for the VWC domain of CCN2 in angiogenesis, HUVEC cells were treated with CCN2ΔVTC and tube formation was measured. Full length CCN2 and CCN2 containing deletions of the CT and TSP1 domains (CCN2ΔTC) caused a similar increase in tube formation which was blocked by co-treatment with GDF-15. After deletion of the VWC domain (CCN2ΔVTC) tube formation was not observed (Supplementary Fig. 3). This finding further supports the role of the VWC domain in the pro-angiogenic function of CCN factors [Lin et al., 2003; Chen et al., 2004].

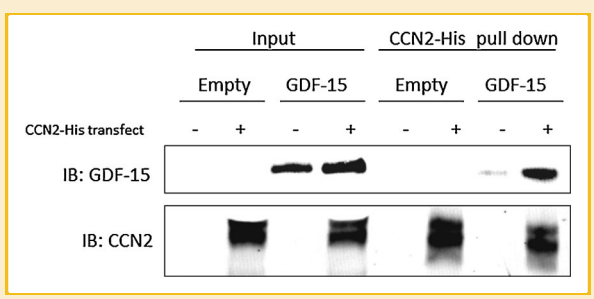

Fig. 2. Biochemical interaction between GDF-15 and CCN2. Conditioned medium collected from PC3 cells coexpressing GDF-15 and His tagged CCN2 (CCN2-His) was used in biochemical pull-down assays. Media were incubated with Ni-NTA beads for 1 h at 4°C to pull down CCN2-His-GDF-15 complex and subjected to immunoblot (IB) analysis for GDF-15 and CCN2. The experiment was repeated three times and a representative result is shown.

GDF-15 SUPPRESSES CCN2-ACTIVATED $\alpha_V\beta_3$ FOCAL ADHESIONS

CCN2 activates integrin signaling in numerous cell types [Jedsadayanmata et al., 1999; Chen et al., 2001; Schober et al., 2002; Tan et al., 2009; Morrison et al., 2010]. Promotion of angiogenesis by CCN2 requires activation of $\alpha_V\beta_3$ integrins which are expressed on the surface of endothelial cells [Lin et al., 2003; Chen et al., 2004]. Upon activation, $\alpha_V\beta_3$ integrins are involved in the formation of signaling complexes at the site of focal adhesions [Jockusch et al., 1995]. To determine if GDF-15 affects CCN2-mediated formation of $\alpha_V\beta_3$ integrin complexes, HUVEC cells were plated on Matrigel and treated with conditioned media containing either CCN2 or GDF-15. Cells were imaged by immunofluorescence using an antibody against the $\alpha_V\beta_3$ integrin heterodimer and phalloidin was used as a counterstain to highlight the cell periphery. $\alpha_V\beta_3$ punctae formation was increased after treatment with CCN2 (Fig. 4). Punctae formation was not observed with CCN2 plus GDF-15 co-treatment indicating that formation of $\alpha_V\beta_3$ complexes are suppressed by GDF-15.

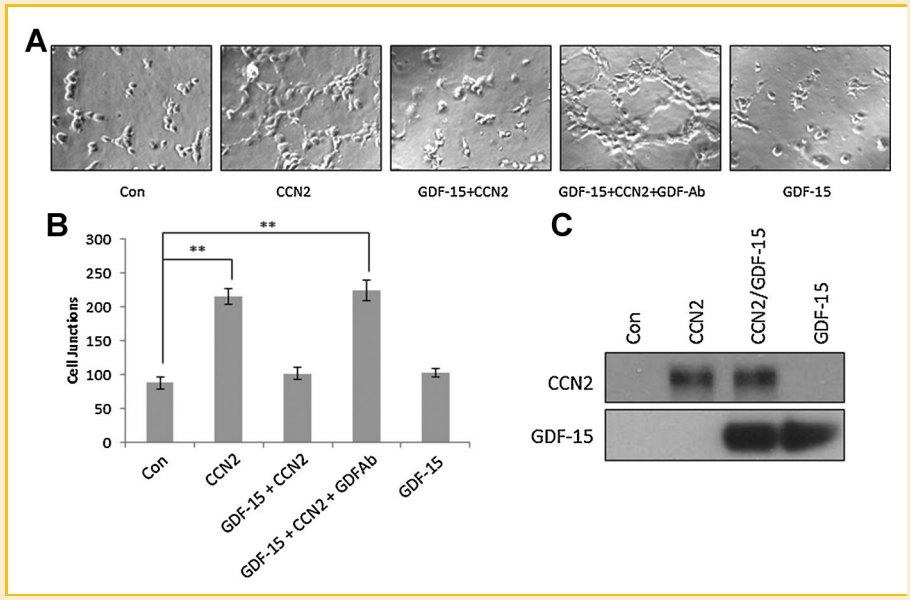

Fig. 3. GDF-15 suppresses CCN2-activated angiogenesis in vitro. A: Tube formation of HUVEC cells plated on basement membrane extract. The cells were incubated with conditioned media from PC3 cells transfected with empty vector (Con), CCN2, GDF-15, or a mixture of media from cells expressing GDF-15 and CCN2 (GDF-15 + CCN2). Blocking antibody against GDF-15 (GDF-Ab) was incubated with media for 20 min before addition of endothelial cells. The images were captured at 200× magnification. The experiment was repeated four times and a representative composite image from one experiment is shown. B: The total number of cell junctions counted in five fields per condition at 100× magnification was quantified. **Significant *P*-value with 99% confidence. *P*-values were calculated using Students *t*-test comparing indicated peak to control. C: Immunoblot showing expression of CCN2 and GDF-15 in conditioned media.

GDF-15 INHIBITS CCN2-MEDIATED FAK ACTIVATION IN HUVEC CELLS

Integrin activation by CCN2 is accompanied by phosphorylation of the tyrosine kinase, focal adhesion kinase (FAK) [Tan et al., 2009]. FAK activates pro-migratory signaling during cell movement and is activated in migrating endothelial cells [Orr and Murphy-Ullrich, 2004; Wary et al., 2012; Zebda et al., 2012]. HUVEC cells were treated with GDF-15 and CCN2 conditioned media and phosphorylation of FAK at tyrosine residue 861 was examined (Fig. 5). CCN2 promoted FAK phosphorylation which was blocked upon co-treatment with GDF-15 (Fig. 5). This result suggests that GDF-15 inhibits CCN2-activated FAK in endothelial cells providing a signaling pathway affected by this interaction which may contribute to the anti-angiogenic properties of GDF-15.

To determine if GDF-15 affects CCN2-activated integrin signaling in fibroblasts, GDF-15 and CCN2 were expressed in NIH-3T3 cells and phosphorylation of FAK was measured. Phosphorylation of FAK was increased with CCN2 expression; however, co-expression with GDF-15 did not alter FAK phosphorylation (Supplementary Fig. 4). This suggests that GDF-15 suppresses CCN2-integrin signaling in endothelial cells, but does not affect CCN2-mediated integrin signaling in fibroblasts.

DISCUSSION

GDF-15 is a member of the TGFβ family suggesting that it may activate the classical TGFβ signaling pathway through Smad phosphorylation. We did not observe GDF-15-mediated Smad phosphorylation using a variety of experimental conditions and cell types (unpublished data), suggesting that GDF-15 does not signal through the canonical TGFβ signaling pathway. This indicated that GDF-15 may signal through an alternate, non-TGFβ receptor mediated mechanism.

To gain insight into a potential alternate signaling mechanism for GDF-15, proteins that interact with GDF-15 were identified in a yeast two-hybrid screen. Using this technique, CCN2 was identified as a GDF-15 binding partner. Some controversy exists over the use of secreted proteins in yeast two-hybrid assays which are carried out using intracellular expression of binding partners in yeast. Despite this, CCN2 interaction with cysteine knot containing secreted proteins was determined using yeast two-hybrid analysis and confirmed biochemically [Pi et al., 2012]. Thus, the yeast two-hybrid system can be applied to interaction studies with secreted proteins. To validate our yeast two-hybrid data for GDF-15:CCN2 interaction, we carried out binding assays after secretion of these proteins from PC3 prostate cancer cells. These biochemical analyses revealed that GDF-15 and CCN2 were complexed after secretion from PC3 cells (Fig. 2).

CCN2 contains four functional domains through which it carries out distinct signaling activities [Lau and Lam, 1999; Chen et al., 2001, 2004; Abreu et al., 2002; Mercurio et al., 2004; Perbal, 2004; Chu et al., 2008; Chen and Lau, 2009; Dhar and Ray, 2010; Kular et al., 2011]. To gain insight into the CCN2-mediated signaling pathway(s) potentially affected by this interaction, the GDF-15:CCN2 interaction domain was mapped. The VWC domain of

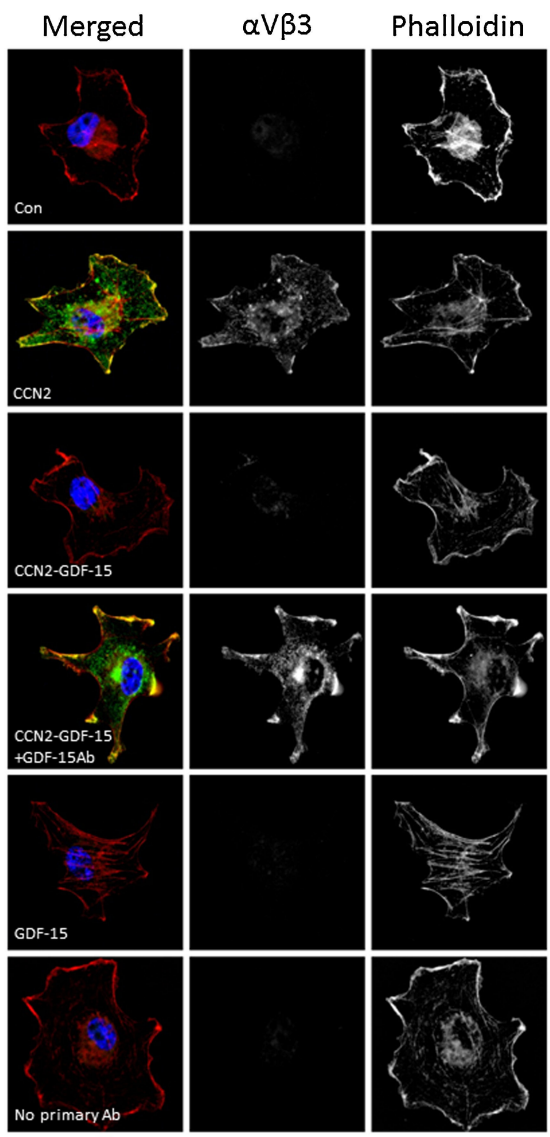

Fig. 4. GDF-15 inhibits CCN2-mediated $\alpha_V\beta_3$ integrin clustering. Immunofluorescent staining of HUVEC cells plated on matrigel-coated coverslips were stained using DAPI (blue) and monoclonal antibody against $\alpha_V\beta_3$ integrin heterodimers (green). Cells were counterstained with phalloidin (red) to highlight the cell periphery. Cells were imaged at 600× and digitally deconvolved using Slidebook software as described in Materials and Methods Section. Representative images are shown taken from at least five images obtained per condition. The experiment was repeated three times.

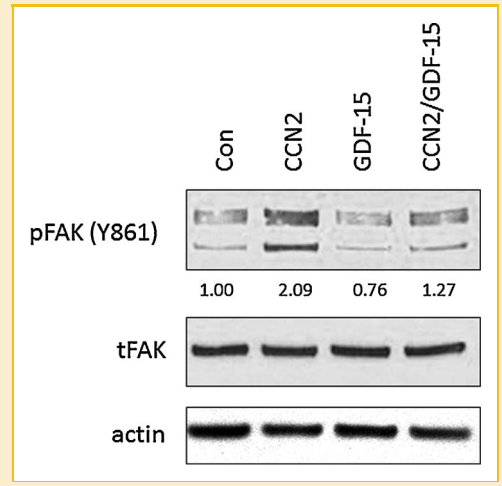

Fig. 5. GDF-15 inhibits CCN2-mediated phosphorylation of focal adhesion kinase. Phosphorylated FAK (pFAK) at tyrosine 861 (Y861) was analyzed by immunoblot using whole cell extracts from HUVEC cells treated with conditioned media from PC3 transfected with empty vector control (Con), or expression vectors for CCN2 or GDF-15. HUVEC cells were also treated with a combination of media containing bot GDF-15 and CCN2 (CCN2/GDF-15). Numbers indicate quantification of the band intensities normalizing pFAK signal to total FAK (tFAK) signal. The experiment was repeated three times and a representative result shown.

CCN2 was identified as the binding domain for GDF-15:CCN2 protein interaction (Fig. 1 and Table I). Two members of the TGFβ family have previously been reported to bind CCN2 through the VWC domain, TGFβ1 and BMP4 [Abreu et al., 2002]. Here, we report a novel interaction with a third TGFβ family member, GDF-15.

CCN proteins carry out pro-angiogenic functions in endothelial cells through their VWC domains [Babic et al., 1999; Lau and Lam, 1999; Shimo et al., 1999; Brigstock, 2002; Lin et al., 2003; Chen et al., 2004]. CCN2 interaction with the cysteine knot-containing protein Slit3 was recently shown to promote angiogenesis in vitro and is associated with tumor angiogenesis in vivo [Pi et al., 2012]. Because GDF-15 was originally classified as a member of the TGFβ family of proteins due to it containing a cysteine knot motif, GDF-15 binding to CCN2 is consistent with an emerging theme describing CCN2 interactions with cysteine knot containing proteins. Considering GDF-15 interaction with the VWC domain of CCN2 and reports describing cysteine knot proteins modulating CCN2-mediated angiogenesis, we reasoned that GDF-15:CCN2 interaction may affect the pro-angiogenic role of CCN2. An in vitro angiogenesis assay revealed that GDF-15 suppresses CCN2-mediated angiogenesis in HUVEC cells (Fig. 3) providing a novel role for this interaction.

CCN2 promotion of angiogenesis requires activation of $\alpha_V\beta_3$ integrins, [Babic et al., 1999] which is mediated by the VWC domain in CCN family members [Lin et al., 2003; Chen et al., 2004] and is the site of GDF-15:CCN2 binding. Upon activation, integrins form complexes at the site of focal adhesions [Jockusch et al., 1995; Anthis and Campbell, 2011; Huttenlocher and Horwitz, 2011]. In our studies, GDF-15 suppressed CCN2-mediated $\alpha_V\beta_3$ complex formation (Fig. 4), suggesting a potential signaling pathway affected downstream of this interaction. In endothelial cells, CCN2-mediated activation of integrins is accompanied by phosphorylation of FAK [Tan et al., 2009]. GDF-15 suppressed CCN2-mediated FAK phosphorylation in HUVEC cells (Fig. 5) revealing a novel signaling mechanism downstream of this interaction. Future work will address the requirement for FAK activation in GDF-15 suppression of angiogenesis.

Our data indicating that GDF-15 inhibits integrin signaling in HUVEC cells raises the possibility that GDF-15 may inhibit CCN2

integrin activation in other cell types. Recently, GDF-15 was reported to suppress integrin signaling in polymorphonuclear leukocytes (PMNs), supporting an anti-inflammatory role described for GDF-15 in murine cardiovascular tissue [Kempf et al., 2011]. Mechanistic details for integrin suppression by GDF-15 in leukocytes have not been described. CCN2 is reported to activate integrins in leukocytes; however, the role of GDF-15:CCN2 binding in the inhibition of integrins in PMNs by GDF-15 has not been established.

CCN2-mediated activation of FAK was inhibited by GDF-15 in endothelial cells but not in fibroblasts (Fig. 5 and Supplementary Fig. 4). CCN2 activates $\alpha_6\beta_1$ integrins in fibroblasts through the TSP1 domain [Heng et al., 2006] which is not bound by GDF-15. $\alpha_V\beta_3$ integrin activation in endothelial cells is carried out through the VWC domain in CCN family members [Lin et al., 2003; Chen et al., 2004] which is the site of GDF-15:CCN2 binding. Our data suggests that GDF-15 blocks the VWC domain of CCN2 from activating $\alpha_V\beta_3$ integrins expressed on the surface of HUVEC cells. CCN2 activates other integrin subtypes through the TSP1 and CT domains [Jedsadayanmata et al., 1999; Chen et al., 2001; Schober et al., 2002; Tan et al., 2009; Morrison et al., 2010]. In light of our domain mapping and signaling data, GDF-15 suppression of integrins may not occur in cells expressing integrin subtypes which are not activated by the VWC domain of CCN2.

Expression of $\alpha_V\beta_3$ integrins is increased in invasive and metastatic tumors compared to normal epithelial or non-malignant tumors [Sabbah et al., 2008; Auzzas et al., 2010; Desgrosellier and Cheresh, 2010; Tabatabai et al., 2010; Schneider et al., 2011]. Inhibition of $\alpha_V\beta_3$ integrins in tumor cells results in decreased proliferation, anchorage independent growth, angiogenic function, and metastatic growth [Kumar, 2003; Nemeth et al., 2003; Mulgrew et al., 2006; McCabe et al., 2007; Sun et al., 2007; Desgrosellier et al., 2009]. The $\alpha_V\beta_3$ inhibitor, cilengitide, is currently in phase III clinical trials for treatment of in glioblastoma multiforme where it was found to increase survival with low toxicity [Reardon et al., 2008; Desgrosellier and Cheresh, 2010; Gilbert et al., 2012]. These promising results highlight the efficacy of targeted therapy against $\alpha_V\beta_3$ in tumors. GDF-15:CCN2 interaction provides a novel mechanism for $\alpha_V\beta_3$ inhibition. GDF-15:CCN2 studies in cancer cells may establish a new role for this interaction in the context of $\alpha_V\beta_3$ driven tumors; however, in vivo studies are needed to define this role.

CCN2 is a promoter of tumor angiogenesis in breast cancer and fibrosarcoma cells [Shimo et al., 2001b; Chien et al., 2011]. CCN2 promoted tumor growth and angiogenesis when expressed in the stroma of prostate xenografts [Yang et al., 2005]. Our data suggests that GDF-15 may play a role in angiogenesis; however, its role in tumor angiogenesis has not been determined. In vivo studies will be essential for determining the role of GDF-15:CCN2 interaction in tumor angiogenesis. A recent report indicated that GDF-15 promotes angiogenesis through p53 and HIF-1α in HUVEC cells grown under hypoxic conditions [Song et al., 2012]. This finding highlights our observation that additional factors and/or conditions are involved in GDF-15 regulated angiogenesis (i.e., CCN2 or hypoxia). Modulation of additional signaling proteins (non-CCN2 dependent) by GDF-15 and/or microenvironmental conditions provides a possible explanation for the conflicting reports implicating GDF-15 as an anti- and pro-angiogenic regulator.

Interaction between epithelial derived GDF-15 and stromal derived CCN2 may provide a novel mechanism for stromal–epithelial signaling. Further studies are needed to define the role of this interaction in vivo. Suppression of CCN2-activated angiogenesis in vitro demonstrates a novel role for GDF-15 in cellular physiology. The observed inhibition of CCN2-mediated FAK and integrin signaling provides a heretofore undescribed signaling mechanism for GDF-15. Future investigations of GDF-15:CCN2 interaction should provide insight into the functional role of these factors during tumorigenesis and other disease states.

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