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Title page

Connective tissue growth factor induces renal fibrosis via epidermal growth factor receptor activation

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ABSTRACT

Connective tissue growth factor (CCN2/CTGF) is a matricellular protein overexpressed in progressive human renal diseases, mainly in fibrotic areas. *In vitro* studies have demonstrated that CCN2 regulates extracellular matrix proteins production (ECM) and epithelial mesenchymal transition (EMT) and therefore could contribute to renal fibrosis. CCN2 blockade ameliorates experimental renal damage, including diminution of ECM accumulation. We have described that CCN2 and its C-terminal degradation product CCN2(IV), bind to epidermal growth factor receptor (EGFR) to modulate renal inflammation. However, the receptor involved in CCN2 profibrotic actions has not been described so far. Using a murine model of systemic administration of CCN2(IV) we have unveiled a fibrotic response in the kidney that was diminished by EGFR blockade. Additionally, in CCN2-conditional knockout mice, renal fibrosis elicited by folic acid-induced renal damage was prevented, linked to inhibition of EGFR pathway activation. Our *in vitro* studies demonstrated a direct effect of CCN2 via the EGFR pathway on ECM production by fibroblasts and the induction of EMT in tubular epithelial cells. Our studies clearly show that the EGFR regulates CCN2 fibrotic signaling in the kidney and suggest that EGFR pathway blockade could be a potential therapeutic option to block CCN2-mediated profibrotic effects in renal diseases.

INTRODUCTION

Regardless of the underlying etiology, most forms of chronic kidney disease (CKD) are characterized by excessive deposition of extracellular matrix proteins (ECM) that replaces the normal tissue by fibrotic scars [1]. Despite the intensive research, our current understanding of the precise mechanism of fibrosis remains limited. Most of the studies have been focused on the later stages of the fibrotic pathway, investigating fibrogenic cytokines, such as TGF-β, on the mechanisms of tubular damage, including G2/M phase cell growth arrest and phenotypic changes involved in epithelial to mesenchymal transition (EMT), and on myofibroblast activation and proliferation [2-4].

Connective tissue growth factor (CCN2/CTGF/Cyr61/Nov) is a 38-kDa cysteine-rich secreted protein, member of the CCN family of matricellular proteins. CCN2 is a fibrogenic cytokine [5-10] that has been identified as a downstream mediator of the pro-fibrogenic effects of TGF-β and other factors, such as angiotensin II (AngII) [11-14], and it has been suggested as a potential anti-fibrotic target in different pathologies [15-16]. In human biopsies of different renal pathologies and in experimental models of kidney injury, renal CCN2 overexpression was correlated with cellular proliferation and ECM accumulation, both in glomerular and interstitial areas [6,12,17]. In cultured cells, recombinant CCN2 and its C-terminal degradation fragment of 10 kDa, also named CCN2(IV), significantly increase ECM production [18-21]. Moreover, CCN2 is one of the most important inducers of EMT [22-24], acting also as a downstream mediator of TGF-β and AngII effects [25]. Both full-length CCN2 and CCN2(IV) cause EMT of tubular epithelial cells, whereas the N-terminal domain does not [24]. Inhibition of endogenous CCN2 by antisense oligonucleotides or gene silencing was beneficial for experimental renal diseases, including unilateral ureteral obstruction model, diabetic nephropathy, and uninephrectomized TGF-β1 transgenic mice [26-29]. These data suggest that CCN2 could be an important target for the treatment of fibrotic disorders and renal diseases.

We have recently reported that epidermal growth factor receptor (EGFR) is a functional CCN2 receptor in the kidney and in aorta linked to the regulation of inflammation [30-32]. CCN2, as well as other matricellular proteins, can bind to other receptors, including TrKa, integrins and heparan sulphate proteoglycans. Additionally, CCN2 can interact with growth factors, such as TGF-β and VEGF, and modify their function [33-36]. In the present work, we have found that CCN2 via EGFR pathway activation regulates fibrosis in the kidney and in cultured renal cells. Moreover, CCN2 induces EMT via EGFR. This suggests that EGFR pathway blockade could be a good therapeutic option to block CCN2-mediated profibrotic effects in renal diseases and, potentially, in other pathological conditions.

Materials and Methods

Animal models

All the procedures on animals were performed according to the European Community and Instituto de Investigación Sanitaria Fundación Jiménez Díaz Animal Research Ethical Committee guidelines.

Systemic administration of CCN2

C57BL/6 male mice (9–12 weeks old, 20 g; Harlan Interfauna Iberica) received a single intraperitoneal injection of 2.5 ng/g of body weight recombinant CCN2(IV) dissolved in saline as previously described [37,38], and were studied 10 days later (n=8–10 mice per group). To block EGFR pathway activation, animals were treated with erlotinib (40 mg/kg/day) or its vehicle (10% ethanol) starting 24 h before CCN2(IV) injection (n= 8–10 mice per group). Control animals were injected with vehicle (10% ethanol). Mice were sacrificed under anaesthesia (Ketamine-HCl/Xylazine-HCl) and then kidneys were perfused in situ with cold saline before removal.

Folic Acid-induced renal injury

Folic acid nephropathy is a model of kidney tubulointerstitial injury progressing to kidney fibrosis [39,40]. Conditional CCN2 deficient mice (CTGFfl/fl-R26CreER) were developed as described [41]. To induce CCN2 gene deletion, mice received 4 injections of 10 mg/ml tamoxifen in corn oil; i.p., over 7 days, followed by a two-week washout period counting from the last injection. Control mice were injected with corn oil. Then, mice were divided in two groups: one received a single intraperitoneal injection of 250 mg/kg folic acid and the other vehicle (0.3 mol/l sodium bicarbonate), and were sacrificed 7 days later, at the time renal fibrosis develops. No renal lesions were observed in corn oil+vehicle (vehicle group, considered as control) or tamoxifen+vehicle (data not shown) groups.

Cultured cells

Reagents: Recombinant proteins: CCN2(IV) (10-50 ng/mL; Preprotech), TGF-β1 (10 ng/mL; Preprotech). Inhibitors (preincubation of 1 hour): Erlotinib; EGFR kinase inhibitor (100 nmol/L, Vichem), Tyrphostin AG 1478 (100 nmol/L, Alomone Labs), ERK inhibitor U0126 (10 µmol/L, Promega); NF-KB inhibitor parthenolide (10 µmol/L, Sigma) and JAK inhibitor I (sc-204021) (250 nmol/L, Santa Cruz Biotechnology). DMSO, used as a solvent in some cases, had no effect on cell viability or on gene expression levels (not shown).

Murine tubular epithelial cells (MCT cell line) cells are a well-characterized line of murine proximal tubular epithelial cells, kindly donated by Eric Neilson (Vanderbilt University) [38]. Cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) at 37ºC in 5% CO_2 atmosphere. HK-2 cells (immortalized human tubular epithelial cell line, HK2; ATCC CRL-2190 cell line) were grown in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine (Sigma), and hydrocortisone (36 ng/mL) (Sigma) at 37ºC in 5% CO_2. NIH3T3 cells (immortalized fibroblast cell line, ATCC CRL-1658 cell line) were grown in DMEM with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37ºC in 5% CO_2. Subconfluent cells (60,000 cells/cm^2) were incubated with stimuli in serum-free medium (HK-2) or with 1% FBS (MCT or NIH3T3 cells) for 15 min or 48 hours.

Renal histology and immunohistochemistry

Immunohistochemistry was carried out on 3 µm paraffin embedded kidney sections. Sections were deparaffinized and exposed to the PT Link (Dako) with Sodium Citrate Buffer (10 mmol/L, pH 6 or 9 depending on the immunohistochemical marker) for antigen retrieval. After endogenous peroxidase was blocked, sections were incubated with 4% BSA/8% serum in 1× wash buffer 'en vision' (Dako) to eliminate nonspecific protein binding, followed by primary antibodies (table 1) overnight at 4ºC. After washing, they were incubated with anti-IgG secondary biotinylated-conjugated antibodies (Amersham) followed by the avidin-biotin-peroxidase complex (Dako) and 3,3-diaminobenzidine as chromogen. Sections were counterstained with Carazzi's haematoxylin. The intensity of staining was assessed using Image-Pro Plus software. For each sample, processed by duplicate in a blinded manner, the average value was obtained from the analysis of 4 fields (20X objective) as density/mm^2 or percentage stained area vs total analyzed area. Data are expressed as n-fold increase over control mice, as mean ± SEM of 6-10 animals per group. Negative controls included non-specific immunoglobulin and no primary antibody (not shown).

Gene silencing

Gene silencing in cultured cells was performed using either pre-designed siRNA corresponding to EGFR, TrKA and CCN2, or their corresponding scramble siRNAs (Ambion). Subconfluent cells were transfected for 24 h with 25 nmol/L siRNA using 50 nmol/L Lipofectamine RNAiMAX (Invitrogen) or treated only with lipofectamine vehicle, according to the manufacturer's instructions. The cells were then incubated with 10% heat-inactivated FBS for 24 h, followed by additional 24 h in serum-free medium before the experiments.

CCN2 antisense oligonucleotides

CCN2 gene blockade in cultured cells was achieved using antisense oligonucleotides against CCN2 (20 ng/mL; Metabion) (DNA AS-oligo CCN2 sequence: 5´-TAC TGG CGG CGG TCA T-3´) or their corresponding sense oligonucleotide for CCN2 (20 ng/mL; Metabion) (DNA S-oligo CCN2 sequence: 5´-ATG ACC GCC GCC AGT A-3´). Subconfluent cells were preincubated for 1h with 20 ng/mL antisense or sense oligonucleotide against CCN2. Cells were then incubated with stimuli.

Protein studies

Proteins were obtained from cells or mouse kidneys using lysis buffer (50 mmol/L Tris-HCl, 150 mol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X-100, 0.3% IGEPAL, 10 µl/ml proteinase inhibitor cocktail, 0.2 mmol/L PMSF, and 0.2 mmol/L orthovanadate). The BCA method was used to determine protein content. For Western blotting, cell (25 µg/lane) protein extracts were separated on 6%–12% polyacrylamide-SDS gels under reducing conditions. Samples were then transferred onto nitrocellulose membranes (BioRad), blocked with TBS/5%

non-fat milk/0.05% Tween-20, and incubated overnight at 4ºC with the corresponding antibodies (table 1). Membranes were subsequently incubated with peroxidase-conjugated IgG secondary antibody and developed using an ECL chemiluminescence kit (Amersham). Loading controls of total protein extracts were performed using an anti-GAPDH or anti-alpha tubulin antibodies (1:10000; Chemicon). The loading control for soluble proteins (cell supernatants) was Ponceau S staining, and the albumin band (67 KDa) was used for quantification of the loading control. This staining binds to the positively charged amino groups and non-covalently to non-polar regions of the protein in the PVDF membrane. Autoradiographs were scanned using the Gel Doc™ EZ imager and analyzed with the Image Lab 3.0 software (BioRad).

Immunofluorescence for EMT markers

Cells grown on coverslips were stimulated with the agonists, and then fixed in Merckofix (Merck, Whitehouse Station, NJ, USA) and permeabilized with 0.1% Triton-X100 for 2 min. After blocking with 10% BSA and 10% FBS for 1 h, they were incubated with several primary antibodies (table 1) for 1 h, followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1/200), for 1 h. Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40-88 (Sigma-Aldrich) and examined by a Leica DM-IRB confocal microscope.

Gene expression studies

Total RNA was isolated from cells and mouse kidney samples with Trizol (Invitrogen). The cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems) using 2 µg total RNA primed with random hexamer primers. Multiplex Real-Time PCR was performed using Applied Biosystems expression assays: Fibronectin Mm01256744_m1, Type I Collagen Mm00483888_m1. Data were normalized to 18S; 4210893E (VIC) and GAPDH Mm99999915_g1 (VIC). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value. Results are expressed in copy numbers, calculated relative to unstimulated cells or control mice, after normalization against 18S or GAPDH.

Statistical analysis

Results throughout the text are expressed as mean±SEM. Differences between groups were assessed by one-way analysis of variance, followed by post-hoc Bonferroni Dunnett method or Mann-Whitney test, as appropriate. $P<0.05$ was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0, Chicago, IL).

RESULTS

CCN2(IV) via EGFR pathway induces fibrosis in the kidney

We have developed a model of systemic administration of CCN2 in C57BL/6 mice by intraperitoneal injection of CCN2(IV), the recombinant C-terminal fragment of CCN2, as

previously described [30,31,34,35], to investigate the renal effects of this protein. The evaluation of renal lesions by light microscopy showed increased collagen deposition in the interstitial area after 10 days (Figure 1A and C). CCN2(IV)-injected kidneys presented increased ECM proteins levels, as observed by increased fibronectin and type I collagen accumulation mainly in tubulointerstitial areas, as evaluated by immunohistochemistry (Figure 1B and D), and confirmed by analysis of total renal protein levels by Western blot (Figure 1E and 1F). Moreover, increased α-SMA positive cells, a characteristic of activated fibroblasts, were detected in kidneys of CCN2(IV)-injected mice (Figure 1B and 1D).

Next, to investigate whether EGFR was involved in renal fibrosis caused by CCN2, mice were treated with the EGFR kinase inhibitor erlotinib. In CCN2(IV)-injected mice, EGFR blockade prevented renal fibrosis, including collagen deposition, and markedly diminished the number of α-SMA positive fibroblasts (Figure 1).

CCN2 gene deletion prevented renal fibrosis induced by folic acid administration

Mice with tamoxifen-driven global deletion of CCN2 were injected with folic acid (FA) to evaluate renal fibrosis. After 7 days of FA injection wild type mice presented increased renal production of fibronectin, observed by immunohistochemistry (Figure 2A and 2B), and confirmed at the gene and protein levels, by real time PCR and Western blot, respectively (Figure 2C and 2D). Moreover, type I collagen accumulation, mainly in the renal interstitium, was found in FA-injected wild type mice (Figure 2A and 2B), as previously described in other genetic backgrounds [42]. In FA-injected CCN2-deleted mice, renal levels of ECM components, both at mRNA and proteins levels, were similar to control mice (Figure 2C and 2D), suggesting that CCN2 regulates matrix accumulation in FA-induced renal damage.

To evaluate EGFR pathway activation, renal levels of EGFR phosphorylated on tyrosine 1068 were evaluated (Figure 2E). In kidneys of FA-injected mice increased EGFR phosphorylation was found. This was significantly prevented in CCN2-deleted mice that showed levels similar to control mice. These data suggest that CCN2 deletion prevented renal fibrosis associated to EGFR pathway activation in FA-induced renal damage.

CCN2(IV) via EGFR pathway regulates extracellular matrix protein synthesis in cultured renal fibroblasts

The direct effect of EGFR pathway on profibrotic CCN2 actions was further characterized in fibroblasts. In cultured renal fibroblasts, we have previously described that CCN2 and CCN2(IV) activate the EGFR pathway [30] . In murine fibroblasts (NIH3T3 cell line) CCN2 blockade, using a CCN2 antisense oligonucleotide, diminished TGF-β-induced fibronectin protein production (figure 3A). Stimulation of NIH3T3 cells with 50 ng/mL CCN2(IV) for 48 hours increased the production of soluble fibronectin and type I collagen, both important components of the ECM. Preincubation with EGFR kinase inhibitor Erlotinib for 1 hour inhibited CCN2(IV)-induced ECM production in fibroblasts (Figure 3B).

EGFR and TrKA cross-talk has been previously described in CCN2-stimulated tubular

epithelial cells [31] and monocytes [36]. Several studies have shown that K252a, an alkaloid-like kinase inhibitor known to selectively inhibit TrkA kinase activity [43], blocked CCN2(IV)-induced activation of several signaling systems, including MAPKs, and ECM production [33,43]. We found that in cultured fibroblasts, pretreatment with the TrKA inhibitor K252a did not modify CCN2(IV)-induced secretion of fibronectin and type I collagen (Figure 3C). These data suggest that EGFR, but not the TrkA pathway, is involved in CCN2(IV) profibrotic events in cultured cells.

CCN2(IV) via EGFR induced EMT in cultured tubular epithelial cells

Stimulation of murine tubular epithelial cells (MCTs) with 10 ng/mL CCN2(IV) for 48 hours caused an epithelial to a myofibroblast-like phenotype transdifferentiation. The transdifferentiated cells lost the typical cobblestone pattern of an epithelial monolayer, and displayed a spindle-shaped, fibroblast-like morphology, assessed by phase contrast microscopy (Figure 4A). In unstimulated tubular epithelial cells, no staining for the mesenchymal marker α-smooth muscle actin (α-SMA) was observed by confocal microscopy. Treatment with CCN2(IV) for 2 days induced α-SMA-positive microfilaments in the cytoplasm, decreased the epithelial marker zonula occludens-1 (ZO-1), and induced the nuclear localization of Snail (Figure 4B). EMT changes were confirmed by Western blot: CCN2(IV) diminished the epithelial marker E-cadherin, essential for the structural integrity of renal epithelium (Figure 4C).

To evaluate the role of the EGFR pathway, cells were preincubated with 2 specific EGFR kinase inhibitors: erlotinib or AG1478. Both EGFR inhibitors blocked CCN2(IV)-induced changes of the phenotype (Figure 4A) and of EMT-related proteins, as assessed by immunofluorescence and Western blot (Figure 4B and 4C).

CCN2(IV) regulates cell proliferation in the kidney via EGFR

CCN2 induces cell proliferation in different cell types, including cultured renal fibroblasts [44,45]. To investigate whether CCN2 regulates cell proliferation-related events in the kidney, the presence of proliferating cells was evaluated using the marker of proliferating cells PCNA [46]. In the kidney of CCN2(IV)-injected mice, increased number of positive PCNA cells was detected by immunohistochemistry, mainly in tubuloepithelial cells, compared to control mice and to erlotinib-treated mice, as confirmed by Western blot (Figure S1A, B and C). The *in vitro* studies, done in tubular epithelial cells and fibroblasts, also showed that stimulation with CCN2(IV) increased PCNA levels which were lowered by pretreatment with the EGFR kinase inhibitor (Figure S1D and E). These data suggests that CCN2 regulates cell proliferation via EGFR in the kidney, and in cultured renal cells.

CCN2(IV) induces tubular epithelial cell arrest in the G2/M phase of cell cycle via EGFR

In injured kidneys, tubular cells growth-arrested in the G2/M phase of the cell cycle secrete profibrotic factors, therefore contributing to renal fibrosis [47,48]. In CCN2(IV)-injected mice, renal expression levels of specific G2/M proteins, such as Cyclin B1, Cdk1/2 and ARK2

[49,50], were elevated compared to control mice. Moreover, in mice treated with erlotinib, renal levels of those G2/M proteins were significantly reduced (Figure 5 A, B and C). In cultured tubular epithelial cells, CCN2(IV) treatment increased the expression of Cyclin B1 and ARK2, an effect that was abolished in the presence of erlotinib (Figure 5 D and E). Thus, EGFR activation is essential for driving renal epithelial cell cycle arrest at the G2/M phase following exposure to CCN2 *in vivo* and *in vitro*.

STAT3 activation is a key mechanism involved in CCN2-induced renal fibrosis

Activation of the JAK/STAT signaling pathway has been involved in renal fibrosis [51]. In the kidneys of CCN2(IV)-injected mice, STAT3 activation was found, as observed by nuclear translocation of STAT3, mainly detected in tubular cells (Figure 6A and B), as well as by increased renal levels of phosphorylated-STAT3 (Figure 6C), compared to control mice kidneys. This activation was mediated by the EGFR pathway as shown by the lack of STAT3 activation in erlotinib-treated CCN2(IV)-injected mice (Figure 6 A, B and C). Moreover, in cultured tubular epithelial cells and fibroblasts, CCN2(IV) increased STAT3 phosphorylation and this was prevented by EGFR kinase inhibition (Figure 6 D and E). Next, we evaluated whether the JAK/STAT signaling pathway was involved in CCN2-mediated profibrotic effects. In fibroblasts preincubation with a JAK1 inhibitor markedly diminished CCN2(IV)-induced ECM protein overproduction (Figure 6F). These data suggest that the JAK/STAT signaling pathway could participate in CCN2-mediated renal fibrosis.

Evaluation of potential mechanisms involved in CCN2(IV)-induced EMT via EGFR activation: ERK, NF-κB pathway and TrkA crosstalk

In several cell types, CCN2(IV) activates the MAPK cascade [38-41]. We have previously shown that ERK activation is an important EGFR signaling mechanism activated by CCN2(IV) in the kidney and in MCTs cells [30]. Now, we found that the MEK/ERK activation inhibitor, U0126, prevented CCN2(IV)-induced conversion of the epithelial to a myofibroblastic phenotype in MCT cells, as shown by phase contrast microscopy (Figure S2A). Moreover, ERK inhibition blocked CCN2(IV)-induction of the EMT markers α-SMA and ZO-1 (Figure S2B), and restored the levels of the epithelial marker E-cadherin to control values (Figure S2C).

We have previously described that CCN2(IV) activates the classical/canonical NF-κB pathway in the kidney and in cultured MCTs, and this was linked to the regulation of renal inflammation [37]. We have confirmed here that in human tubular epithelial cells CCN2(IV) also activates the NF-κB pathway, as shown by increased levels of phosphorylated p65 NF-κB subunit (Figure S3). Now, the role of EGFR pathway on NF-κB signaling was investigated using a pharmacological and a gene-silencing approach. In CCN2(IV)-treated cells, the EGFR kinase inhibitor erlotinib reduced p65 phosphorylation levels to control values (Figure S3A). Moreover, in cells transfected with an EGFR siRNA, CCN2(IV) failed to increase p65 phosphorylation (Figure S3B).

To evaluate the role of NF-κB signaling in EMT, MCT cells were preincubated with the

NF-κB inhibitor parthenolide. CCN2(IV)-induced fibroblastic phenotypic changes and E-cadherin loss were prevented by parthenolide pretreatment (Figure S2D and G). These results clearly show that CCN2(IV) activates NF-κB signaling via EGFR to modulate EMT in cultured tubular epithelial cells.

EGFR and TrkA activities are mutually influenced in response to CCN2 stimulation in cultured tubular epithelial cells [30]. However, the functional consequences of this crosstalk and downstream signaling pathways were not investigated. Now, we found that in cultured murine tubular epithelial cells, pretreatment with the TrKA inhibitor K252a restored the typical cobblestone pattern of an epithelial monolayer (Figure S2D), and prevented E-cadherin loss caused by CCN2(IV) (Figure S2E). To investigate downstream signaling mechanisms involved in the TrkA pathway, ERK activation was evaluated. Preincubation with K252a diminished CCN2(IV)-induced ERK 1/2 activation (Figure S2F). Finally, the NF-κB pathway was also evaluated. TrKA blockade, via the TrKA inhibitor (K252a) or by gene silencing, did not modify CCN2(IV)-induced p65 phosphorylation (Figure S3C and D), clearly showing that the TrkA pathway is not involved in the activation of the NF-κB pathway in cultured renal cells.

These results demonstrate that the EGFR and TrkA crosstalk regulates EMT by the modulation of ERK signaling. In addition, EGFR-induced NF-κB essentially contributed to CCN2(IV)-induced EMT. However, NF-κB is not involved in ECM production induced by CCN2(IV) and was not regulated by TrkA, showing different downstream mechanisms.

DISCUSSION

The main finding of this paper is that CCN2 regulates the fibrotic process in the kidney via activation of the EGFR signaling pathway. Previous studies have found CCN2 overexpression in fibrotic areas in human and experimental renal diseases [6,12,17]. Preclinical studies have shown that CCN2 blockade by gene silencing or antisense oligonucleotides ameliorates renal damage and diminishes fibrosis [26-29], however these therapeutic strategies are far from being used in humans. Now, using a model of systemic administration of the recombinant C-terminal fragment of CCN2 we have found out a fibrotic response in the kidney, including elevated collagen accumulation, mainly in tubulointerstitial areas, and increased in the number of activated fibroblasts, the main collagen-producing cells, which were prevented by treatment with an EGFR kinase inhibitor. These results have been confirmed *in vitro*. In cultured fibroblasts CCN2(IV), via the EGFR pathway, regulates ECM production. Thus, the observed improvement in experimental renal fibrosis by EGFR inhibition [52-55] may be, at least in part, related to interference with the pro-fibrotic actions of CCN2. In human progressive renal diseases EGFR activation was found in tubulointerstitial areas, in association with CCN2 overexpression and renal fibrosis. In this regard, we have now found that diminishment of experimental renal fibrosis induced by folic-acid administration in CCN2 deleted mice was linked with lower EGFR activation. These data suggest that EGFR pathway inhibition could be a good therapeutic option to block CCN2-mediated profibrotic effects in renal diseases.

CCN2 has been involved in several proliferative disorders [5-10]. Several EGFR ligands

such as EGF, HB-EGF, epiregulin and CCN2, can induce proliferation, migration and invasion in epithelial cells and fibroblasts [44,56-59]. Now, we have found that CCN2 via EGFR signaling activation can regulate cell proliferation in the kidney, as demonstrated by the increased number of positive PCNA proliferating cells. Proliferation of renal interstitial myofibroblasts is a hallmark of renal fibrosis and several studies showed that EGFR activation was associated with proliferation of cultured renal fibroblasts *in vitro* [60], as we have observed here in response to CCN2(IV)/EGFR stimulation.

During renal injury, tubular epithelial cells can dedifferentiate acquiring the capacity of proliferate, migrate and undergo EMT phenotypic changes [61-64]. In CKD patients, activation of EGFR was observed in tubulointerstitial cells within fibrotic areas. CCN2(IV) binding sites in the murine kidney are mainly located in tubular epithelial cells and colocalized with activated EGFR signaling [30]. Interestingly, many proliferating cells in CCN2(IV)-injected mice were tubuloepithelial cells, suggesting that activation of tubular cells by CCN2 is a key mechanism involved in renal damage. In the kidney, the interaction of CCN2 and tubular epithelial cells has been proven to mediate tubular cell hypertrophy and EMT [22-24]. We have previously demonstrated, using CCN2 antisense deoxy-oligonucleotides, that CCN2 is a downstream mediator of AngII-induced *in vitro* EMT [26]. The present findings indicate that EGFR activation by CCN2(IV) can promote EMT in cultured tubular epithelial cells. Although the contribution of EMT to renal fibrosis is a matter of intense debate [65-67], the loss of epithelial properties of the tubular epithelial cells, including permeability and polarity, may result in decreased viability and contribute to renal injury [68]. These EMT-related changes are an initial step in renal damage and an important potential therapeutic target. In cancer progression, EMT is an important process mainly involved in metastases of malignant solid tumors. CCN2 has been involved in tumor progression by inducing EMT. Moreover, CCN2 is preferentially produced in tumor cells, and correlates with poor clinical prognosis in breast tumors [69]. EGFR signaling blockade has emerged as a novel therapeutic option in cancer [70], but the potential role of EGFR in CCN2 actions in cancer will require future studies.

In damaged kidneys, tubular cells can be growth-arrested in the G2/M phase of the cell cycle and acquire a senescence-associated secretory phenotype, characterized by the production of profibrotic factors, another mechanism involved in the development of fibrosis [47,48]. Importantly, CCN2 *in vivo* and *in vitro* induced G2/M arrest of tubular cells, as shown by the evaluation of G2/M regulators cyclin B1, CDK1/2 and ARK2. Erlotinib blocked these changes in the cell cycle, suggesting that EGFR activation by CCN2 is required for renal tubular cell dedifferentiation or proliferation and contributes to epithelial cell arrest in the G2/M phase of the cell cycle.

Previous studies from our group have shown that CCN2 activates STAT3 signaling in isolated human naive CD4+ T cells to regulate Th17 differentiation, contributing to the activation of the Th17 immune response in renal damage [71]. In the present manuscript, we have observed that EGFR blockade inhibited STAT3 activation induced by CCN2(IV) in the kidney and *in vitro*. Emerging evidence suggests that the STAT3 signaling pathway participates in the

fibrotic response in several experimental renal diseases, including obstructive nephropathy and is required for renal fibroblast transformation into myofibroblasts [51,72-74]. The pharmacological blockade of the JAK/STAT pathway *in vitro* inhibited CCN2(IV)-induced production of ECM proteins, supporting the role of STAT-3 in the regulation of fibrosis and suggesting a novel mechanism involved in CCN2 profibrotic responses in the kidney.

Previous studies have described that CCN2 can bind to other membrane receptors besides EGFR. In mesangial cells, CCN2 binds to TrkA [33], and this has been implicated in diabetic nephropathy [75]. In murine cardiomyocytes, the TrkA pharmacological inhibitor K252a blocked profibrotic and proinflammatory effects induced by CCN2 [76]. Some data suggest that Trks could participate in EMT, as described in cancer studies [77]. Accordingly, we have found that the pharmacological TrkA blockade reversed the EMT changes caused by CCN2(IV) in tubular epithelial cells.

CCN2(IV) activates MAPK cascade in several cell types [33,78-80], including murine tubuloepithelial cells [21,37], regulating many cellular responses. In mesangial cells, ERK1/2 mediated CCN2-induced fibrosis, migration, cytoskeleton reorganization and chemokine production [81-83]. In fibroblasts, ERK regulate CCN2-induced expression of α-SMA and type I collagen and myofibroblast proliferation [84,85]. In tubular epithelial cells, ERK is involved in EMT induced by CCN2(IV) [86], and AngII [25]. We have previously shown that ERK is activated as a downstream mechanism of CCN2/EGFR signaling [30], and now, we have described here that an ERK1/2 inhibitor prevented EMT triggered by CCN2(IV). Regarding the EGFR/Trka crosstalk, our data show that ERK activation is a common signaling system between EGFR and TrKa pathways, and also extend previous observations underscoring the importance of the ERK pathway as a key signaling system in EMT.

Previous studies from our group have shown that CCN2 activates the classical NF-κB pathway and this is linked to the regulation of proinflammatory events in the kidney [37] and in the vasculature [31]. In the present manuscript, we have observed that EGFR blockade with a pharmacological inhibitor (erlotinib) or by gene silencing prevented the canonical NF-κB activation induced by CCN2(IV) in cultured tubular epithelial cells. Studies in cancer pointed out that modulation of NF-κB could be a potential therapeutic target due to its effect on to EMT [69,87,88]. Activation of canonical NF-κB regulates Snail, a transcription factor that represses E-cadherin expression, leading to the disruption of epithelial membrane properties and inducing EMT [89]. In tubular epithelial cells, we have found that NF-κB pathway activation is involved in CCN2(IV)-induced EMT, and, additionally, NF-κB activation is mediated by the EGFR pathway. Interestingly, using the same approaches, TrkA blockade did not inhibit NF-κB activation, showing that downstream signaling differs between EGFR and TrKA.

CONCLUSION

Overall our data show that CCN2 regulates fibrotic and proliferative events in the kidney and *in vitro* in fibroblasts and tubular epithelial cells, via the EGFR pathway. These results could contribute to extend the research into other pathological conditions associated with CCN2

overexpression by evaluating the effect of EGFR blockers in different fibrotic and proliferative disorders.

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AUTHOR CONTRIBUTION

All the authors have reviewed the ms and have approved the final version to be published.

SRM contributed to the design of the experiments, acquisition, analysis and interpretation of the all data, and drafted the ms.

JLMP contributed to the acquisition of several data, participating in the development of mice models and analyzing the data.

RRRD and RR D contributed to the acquisition and analyzing of several data.

LF and RG have collaborated in the Conditional CCN2 deficient mice (CTGFfl/fl-R26CreER) studies, design of some experiments and data interpretation.

AO, SM and JE contributed to the critical review of the ms and the financial support of the work.

MRO contributed to the design of the experiments, analysis and interpretation of the all data, draft of the ms and financial support of the experiments.

Statement of competing financial interest: None declared

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FIGURE LEGENDS

Figure 1. Systemic administration of CCN2(IV) in mice caused renal fibrosis that was prevented by EGFR blockade. C57BL/6 mice were i.p. injected with recombinant CCN2(IV) (2.5 ng/mouse) or vehicle (10% ethanol) and sacrificed 10 days later. Some mice were also treated with erlotinib (EGFR kinase inhibitor, 40 mg/kg/day), starting 24 hours before CCN2(IV) administration. In paraffin-embedded kidney sections, renal morphology was evaluated by Masson staining and collagen deposition by Sirius Red staining **(A).** Immunohistochemistry was done using antibodies against ECM proteins fibronectin and type I collagen, and α-SMA, marker of activated fibroblasts **(B).** Figures show a representative mouse from each group. Magnification 400X in figure A and 200X in figure B**.** Quantification of Sirius Red staining **(C)** and immunohistochemistry **(D)** calculated as stained area vs total area. Protein levels were evaluated in total renal extracts by Western blot. α-tubulin levels were used as loading control. Figures **E** and F show several representative mice of each group and the quantification of the Western blot data. Mean ± SEM of 8-10 mice per group. *p<0.05 vs control. # p<0.05 vs CCN2(IV).

Figure 2. CCN2 gene deletion diminished renal fibrosis in folic acid-induced kidney damage. Conditional CCN2 deficient mice (CTGFfl/fl-R26CreER) were injected with corn oil (vehicle), or with tamoxifen to induce CCN2 deletion. Then, mice were divided in two groups

one received a single intraperitoneal injection of folic acid (FA, 250 mg/kg) and the other vehicle (0.3 mol/l sodium bicarbonate), and were sacrificed 7 days later. **(A)** In paraffin-embedded kidney sections, ECM accumulation was measured by immunohistochemistry using antibodies against fibronectin and type I collagen**.** Figures show a representative mouse from each group. Magnification 200X. **(B)** Quantification of fibronectin and type I collagen stained area vs total area. **(C)** RNA was obtained from total renal extracts and gene expression levels were determined by Real Time PCR. Renal total protein levels of control, FA-injected mice and FA-injected CCN2-deleted mice were studied. **(D)** Changes in ECM protein levels were analyzed by Western blot. **(E) CCN2 gene deletion decreased EGFR pathway activation in folic acid-induced renal damage.** Activation of the EGFR pathway was evaluated by changes in phosphorylation levels of EGFR ($pEGFR_{1068}$), analyzed by Western blot. α-tubulin was used as loading control. Figures D and E show representative Western blot and the quantification of the data. Mean ± SEM of 6-8 mice per group. *p<0.05 vs control. # p<0.05 vs FA.

Figure 3. A. CCN2 regulates the production of ECM proteins in cultured fibroblasts. NIH3T3 cells were pre-incubated for 1 hour with 20 ng/mL CCN2 antisense oligonucleotide, to block endogenous CCN2 production, or its corresponding sense oligonucleotide (control) and then stimulated with 10 ng/mL TGF-β1 for 48 hours. Changes in soluble fibronectin protein levels were analyzed by Western blot. Representative autoradiograms are shown. Results are mean ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs TGF-β1 alone. **CCN2(IV) regulates the production of ECM proteins via EGFR activation (B), but independently from TrKA (C).** NIH3T3 cells were pre-incubated for 1 hour with **(B)** 10 μmol/L EGFR kinase inhibitor erlotinib or **(C)** 10 μmol/LTrKA inhibitor K252a and then stimulated with 50 ng/mL CCN2(IV) for 48 hours. Changes in soluble fibronectin and type I collagen proteins were analyzed by Western blot. Representative autoradiograms are shown. Results are mean ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

Figure 4. CCN2(IV) via EGFR promotes epithelial to mesenchymal transition in tubular epithelial cells. MCTs cells were pre-incubated for 1 hour with different EGFR kinase inhibitors: erlotinib (10 μmol/L) or AG 1478 (100 nmol/L). Then, cells were stimulated with CCN2(IV) (10 ng/mL) for 48 hours. **(A)** Phase contrast microscopy analysis of the cell morphology. **(B)** Confocal microscopy analysis of α-SMA, ZO-1 and SNAIL immunofluorescence using specific primary antibodies, and a FITC-labeled secondary IgG was done. This represents the results of 3 independent observations. **(C)** Changes in E-cadherin protein expression were analyzed by Western blot. Representative autoradiograms are shown. Results are data ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

Figure 5. CCN2(IV) regulates renal epithelial cell arrest in the G2/M phase via EGFR. In CCN2(IV)-injected mice treated or not with erlotinib, renal levels of Cyclin B1 and CDK1/2 were measured by immunohistochemistry. Figure A show a representative animal from each group (Magnification 200X), and in B the quantification of the data. **(C)** In the whole kidney protein extracts Cyclin B1 and ARK2 expression levels were evaluated by Western blot. Figure shows several mice of each group and the quantification of the data. Mean ± SEM of 8-10 mice per group. *p<0.05 vs control. # p<0.05 vs CCN2(IV). **(D and E)** MCT cells were pre-incubated for 1 hour with 10 nmol/L EGFR inhibitor Erlotinib and then stimulated with 50 ng/mL CCN2(IV) for 48 hours. Changes in Cyclin B1 and ARK2 protein levels were analyzed by Western blot. Representative autoradiograms are shown. Results expressed as are mean ± SEM from at least 2-4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

Figure 6. CCN2(IV) activates the JAK/STAT3 signaling pathway via EGFR in the kidney and cultured renal cells. In CCN2(IV)-injected mice treated or not with erlotinib, STAT3 activation was evaluated by immunohistochemistry (**A)** or Western Blot (**B).** Figure A shows nuclear localization of STAT3 in tubular cells of CCN2(IV)-injected mice (insert), that was not found in control or erlotinib treated mice. Figure A shows a representative animal from each group. The arrow indicates a positive STAT3 nuclear staining (Magnification 200X) and in B the quantification of the data. **(C)** Representative Western Blot showing increased levels of phosphorylated-STAT-3 and quantification of the data. Mean ± SEM of 8-10 mice per group. *p<0.05 vs control; # p<0.05 vs CCN2(IV). (**D and E***)* Changes in p-STAT3 protein levels expression in cultured tubular MCT cells **(D)** and NIH3T3 fibroblasts **(E)** were analyzed by Western blot. Representative autoradiograms are shown. Results are expressed as mean ±

SEM from at least 2-4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone. (**F) The JAK/STAT pathway regulates extracellular matrix protein synthesis induced by CCN2(IV) in cultured fibroblasts**. NIH3T3 fibroblasts were pre-incubated for 1 hour with 250 nmol/L JAK inhibitor I (JAKi) and then, stimulated with 50 ng/mL CCN2(IV) for 48 hours. Changes in soluble fibronectin protein levels were analyzed by Western blot. Representative autoradiograms are shown. Results are mean ± SEM from at least 2-4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone. Figure 6 **(C)** and Figure S1 **(C)** have the same image as loading control.

Table 1. Antibodies used in Methods. Dilutions and commercial distributor

Antibody	Dilution	Comercial distributor
Immunohistochemistry Antibodies		
Type I collagen	(1:200)	Millipore
Fibronectin	(1:400)	Millipore
α-Smooth muscle actin (α-SMA)	(1:200)	Sigma Aldrich
$pEGFR_{1173}$	(1:200)	Sigma Aldrich
STAT3	(1:200)	Cell Signalling
Cyclin B1	(1:200)	Santa Cruz Biotechnology
Cdk 1/2	(1:200)	Santa Cruz Biotechnology
Western blot Antibodies		
E-cadherin	(1:500)	BD
p-p65	(1:500)	Cell Signalling
$p\text{-}EGFR_{1068}$	(1:250)	Calbiochem
$p\text{-}TrkA_{490}$	(1:1000)	Cell Signalling
p-STAT3	(1:500)	Cell Signalling
fibronectin	(1:400)	Millipore
EGFR	(1:200)	Santa Cruz Biotechnology
PCNA	(1:500)	Santa Cruz Biotechnology
cyclin B1	(1:500)	Santa Cruz Biotechnology
ARK2	(1:500)	Santa Cruz Biotechnology
p-ERK1/2	(1:200)	Santa Cruz Biotechnology
Immunofluorescence Antibodies		
Z0-1	(1:200)	Zymed Laboratories
α-Smooth muscle actin (α-SMA)	(1:200)	ABCam
Snail	(1:200)	Santa Cruz Biotechnology
(FITC)-conjugated secondary antibody	(1:200)	Sigma Aldrich

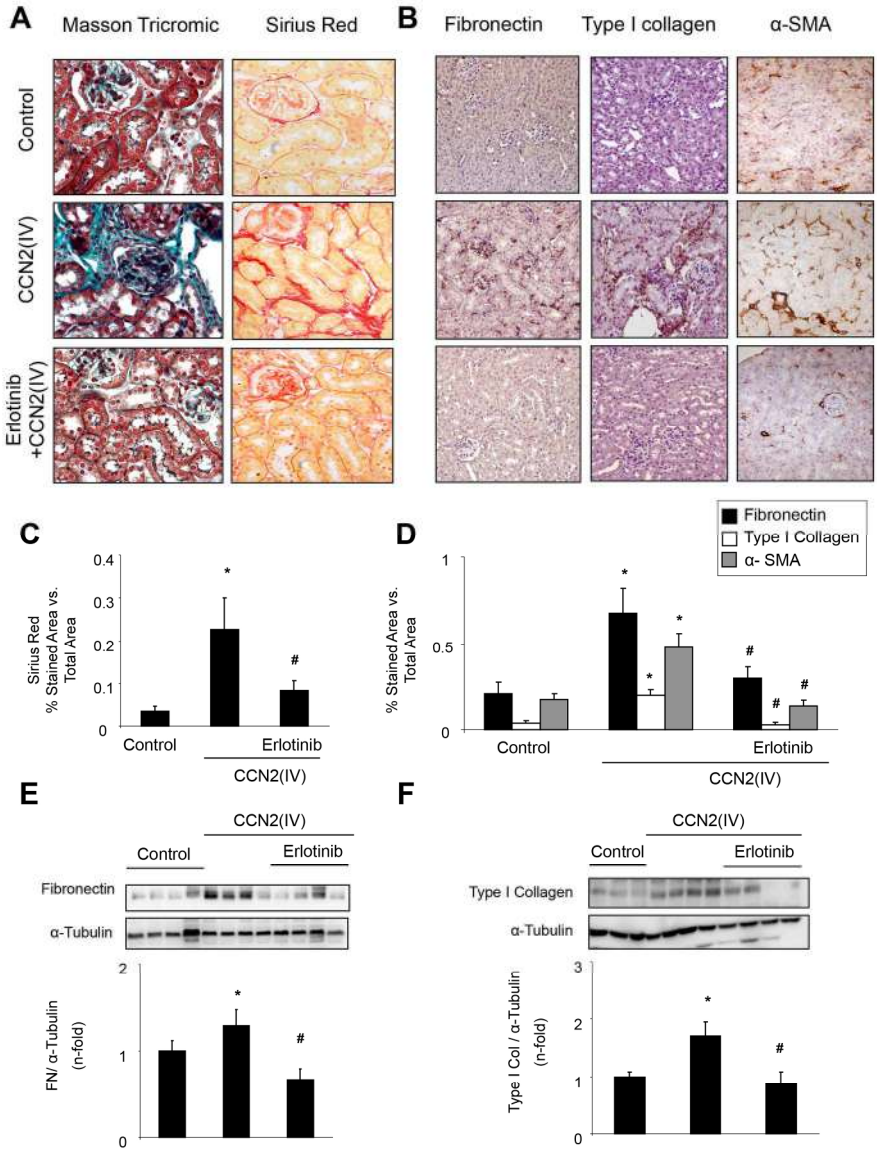

Figure 1

226x302mm (300 x 300 DPI)

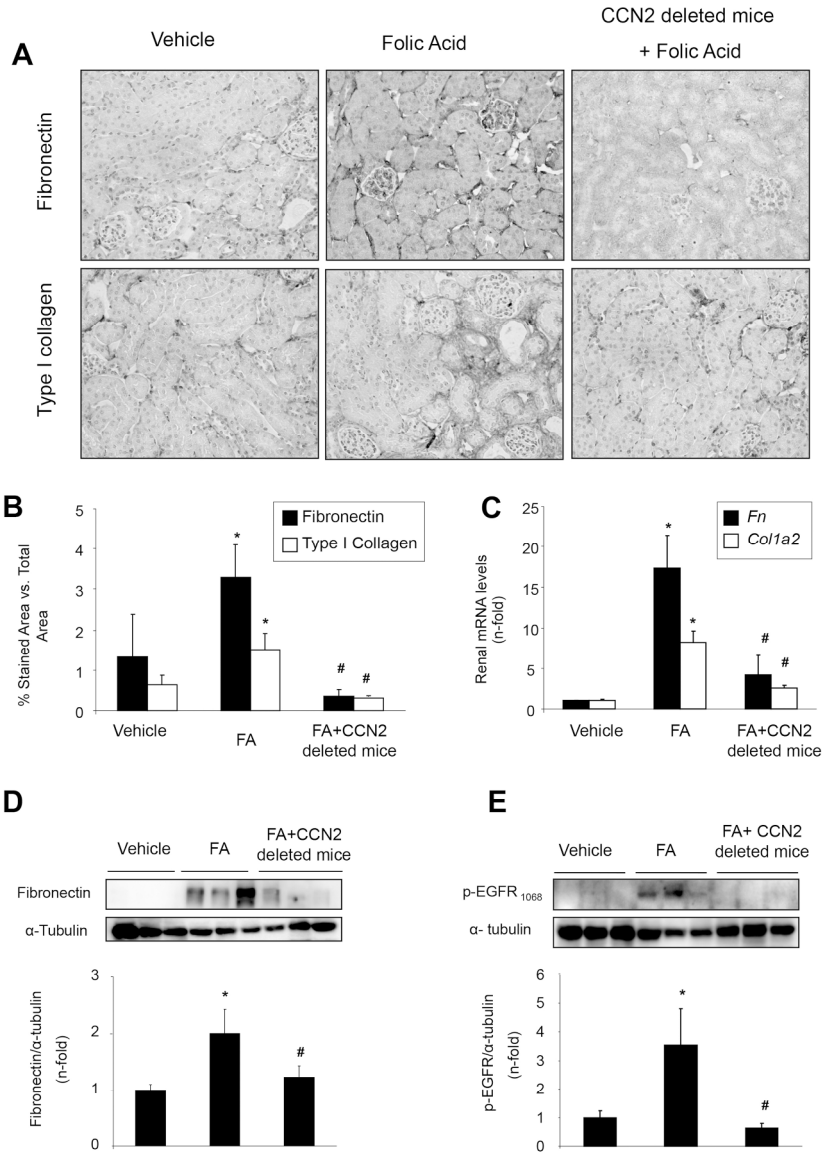

Figure 2

226x302mm (300 x 300 DPI)

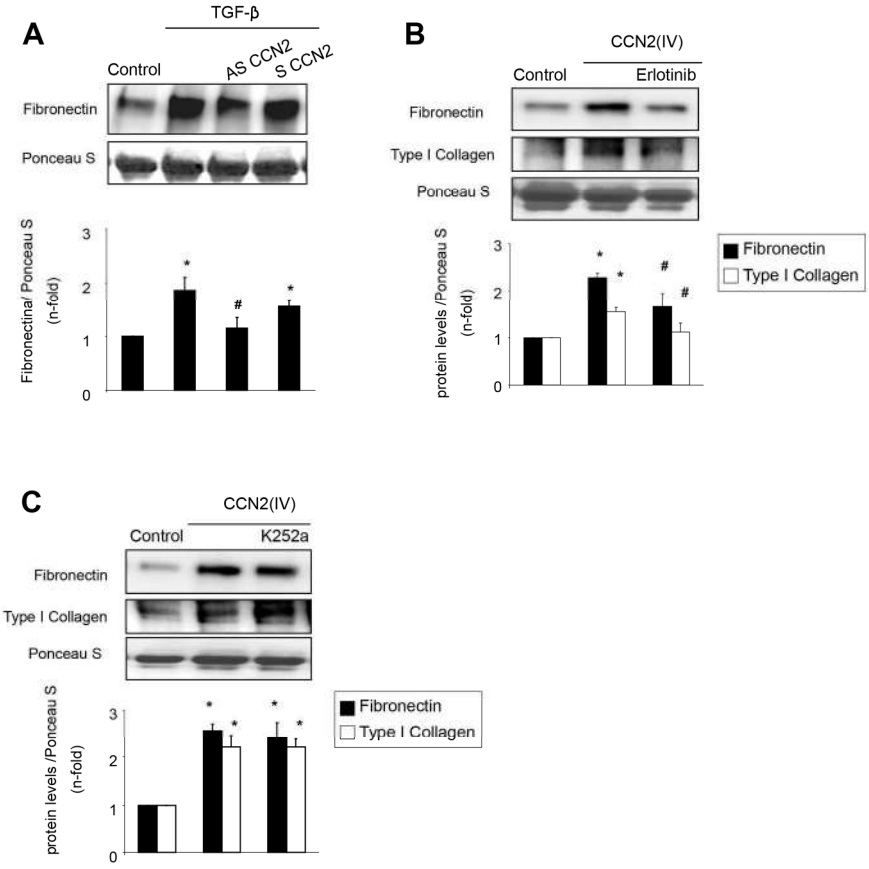

Figure 3

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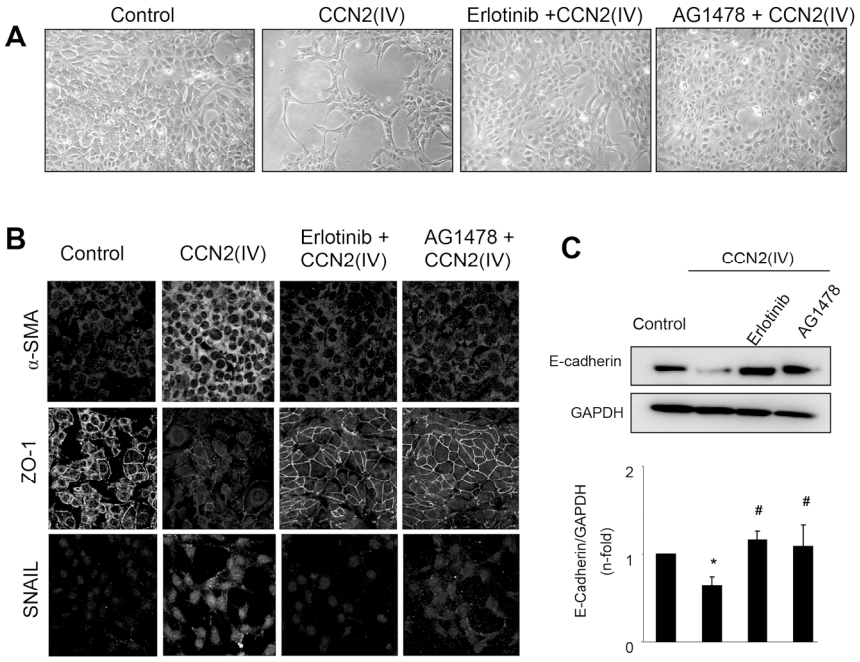

Figure 4

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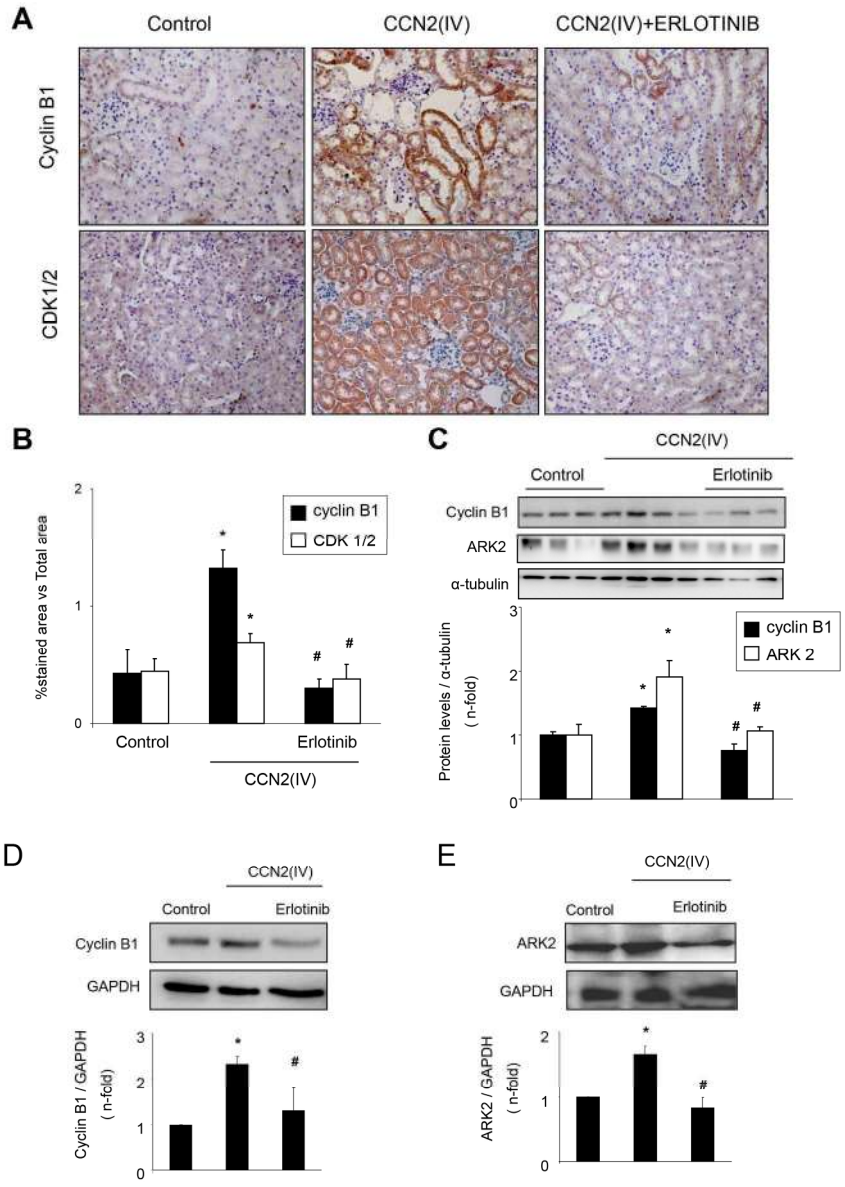

Figure 5

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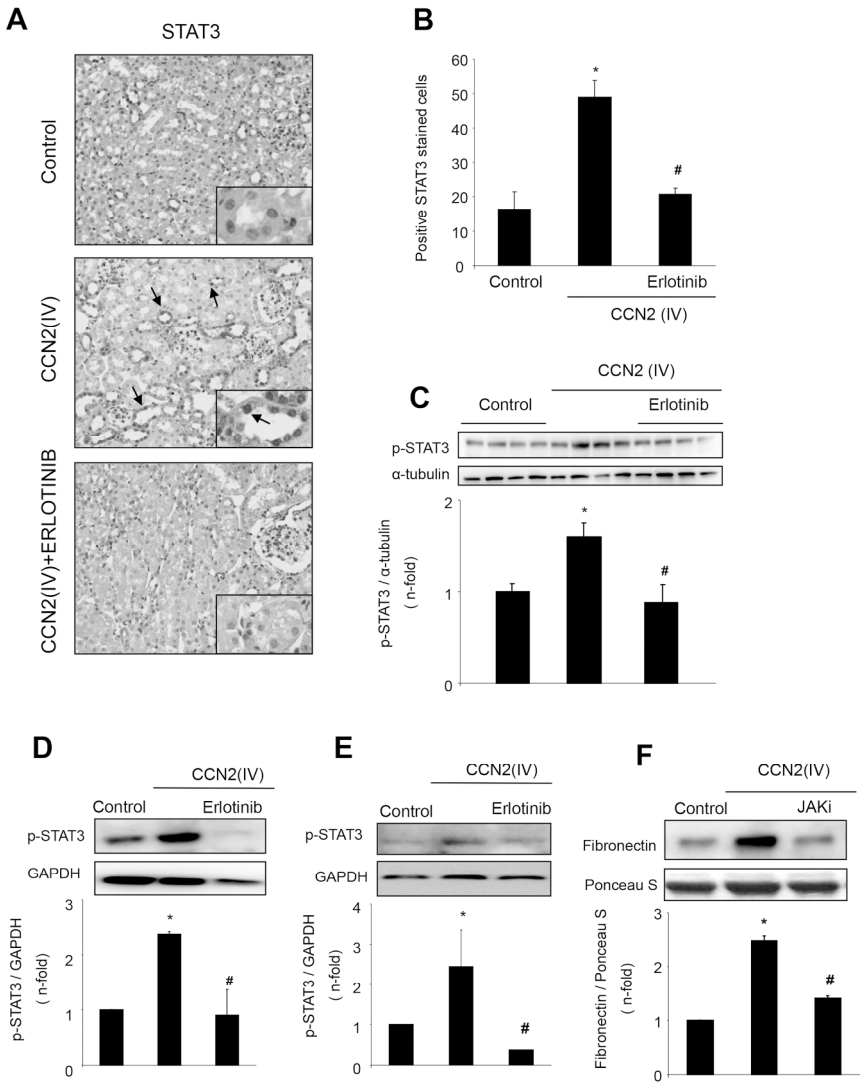

Figure 6

226x302mm (300 x 300 DPI)

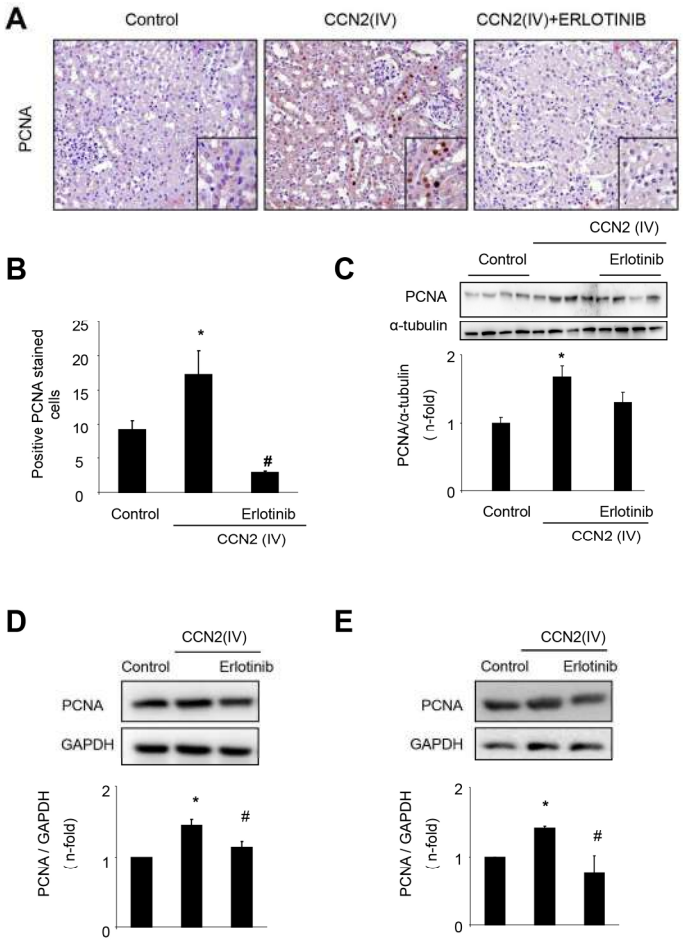

Figure S1. ***CCN2(IV) via EGFR regulates cell proliferation in the kidney and cultured cells.*** C57BL/6 mice were i.p. injected with recombinant CCN2(IV) (2.5 ng/mouse) or vehicle (10% ethanol) and sacrificed 10 days later. Some mice were also treated with erlotinib (EGFR kinase inhibitor, 40 mg/kg/day), starting 24 hours before CCN2(IV) administration. **(A).** In paraffin-embedded kidney sections, renal cell proliferation was evaluated using an antibody against the proliferating marker PCNA. Figure A shows a representative mouse from each group (Magnification 200X) and in **(B)** the quantification of PCNA positive cells. **(C)** Protein levels were evaluated in from total renal extracts by western blot. α-tubulin levels were used as loading control. Figure C shows a representative Western Blot and the quantification of the data. Mean ± SEM of 8-10 mice per group. *p<0.05 vs control. **(D and E)** EGFR pathway blockade reduces PCNA expression induced by CCN2(IV) in tubuloepithelial cells and fibroblasts**.** Cells were pre-incubated for 1 hour with 10 nmol/L EGFR inhibitor Erlotinib and then, stimulated with 50 ng/mL CCN2(IV) for 48 hours. Changes in PCNA protein levels expression were analyzed by Western blot. GAPDH was used as loading control. Representative autoradiograms are shown. Results are mean ± SEM from at least 2-4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone. Note: Figure S1 (D,E) have the same image of loading control that figure 6 (D,E).

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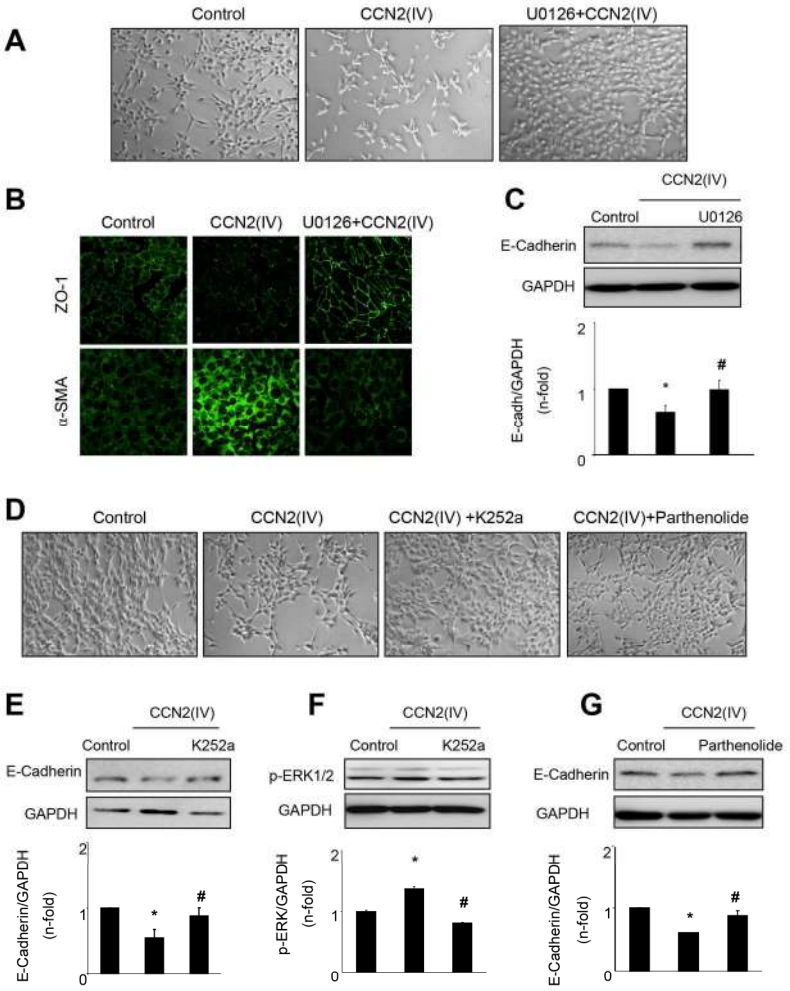

Figure S2. Molecular mechanisms involved in CCN2(IV) induced epithelial to mesenchymal transition in cultured tubular epithelial cells: Role ERK, NF-κB and TRkA activation. MCTs cells were pre-incubated for 1 hour with differeten inhibitors: 10 μmol/L U0126 (an ERK 1/2 inhibitor), 1 μmol/L parthenolide (NF-κB inhibitor) or 10 μmol/L K252a (TrKA inhibitor), and then stimulated with 10 ng/mL CCN2(IV) for 48 hours. Phase contrast microscopy analysis of cell morphology **(A and D)**. Confocal microscopy analysis of α-SMA and ZO-1 immunofluorescence localization. Figure **B** represents the results of 3 independent observations. Changes in E-cadherin **(C, E, G)** and p-ERK 1/2 **(F)** protein expression levels were analyzed by Western blot. GAPDH was used as loading control. Representative autoradiograms are shown. Results are mean ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone.

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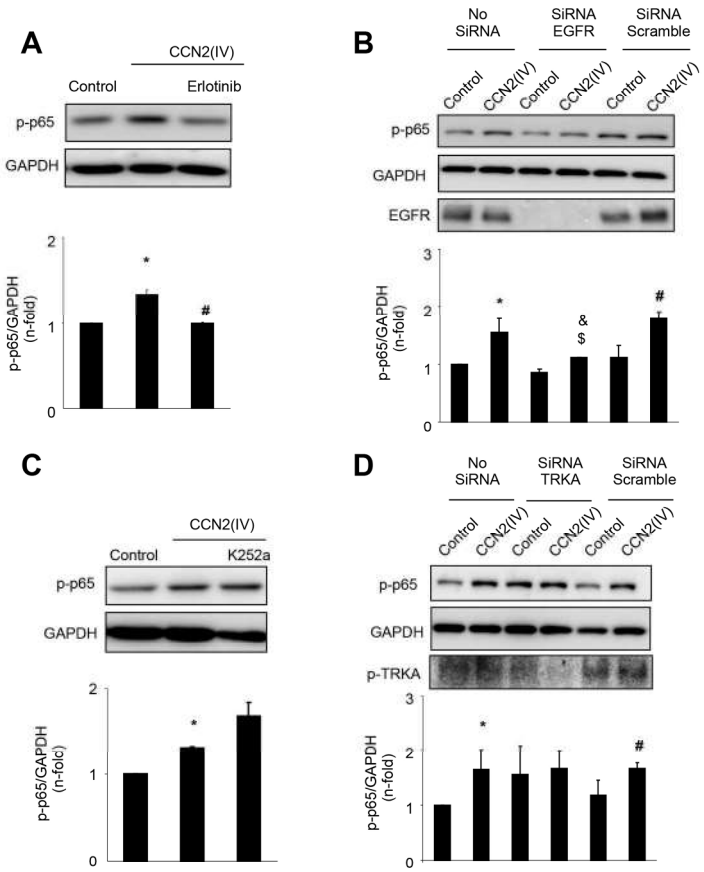

Figure S3. CCN2(IV) activates the NF-κB pathway via EGFR in cultured tubular epithelial cells. (A) Cells were pre-incubated for 1 hour with 10 µmol/L erlotinib (EGFR inhibitor) and then stimulated with 50 ng/mL CCN2(IV) for 15 min. Results are mean ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone. (**B)** HK2 cells were transfected with EGFR siRNA or scrambled siRNA or incubated with transfection reagent alone (no siRNA). Cells were stimulated with 50 ng/ml CCN2(IV) for 15 min. p65 phosphorylation was evaluated by Western blot. GAPDH was used as loading control. Representative Western blot experiments and quantification expressed as p-p65/GAPDH ratio as mean ± SEM of 3 independent experiments *p<0.05 vs control-untransfected (no siRNA). # p<0.05 vs. untreated scramble siRNA-transfected cells. $ p<0.05 vs. CCN2(IV)-treated scramble siRNA-transfected cells. & p<0.05 vs. CCN2(IV)-treated untransfected cells. **CCN2(IV) activates the NF-κB pathway independently of TrKA. (C)** Cells were preincubated for 1 hour with the 10 µmol/L K252a (TrKA inhibitor) and then stimulated with 50 ng/ml CCN2(IV) for 15 min. Results are mean ± SEM from at least 4 independent experiments. *p<0.05 vs control; # p<0.05 vs CCN2(IV) alone. **(D)** Cells were transfected with TrkA siRNA or scrambled siRNA or incubated with transfection reagent alone (non siRNA). Cells were treated with 50 ng/ml CCN2(IV) for 15 min. p65 phosphorylation was evaluated by Western blot using an antibody against p-p65. GAPDH levels were used as loading control. Representative Western blot experiments and quantification expressed as p-p65/GAPDH ratio as mean ± SEM of 3 independent experiments *p<0.05 vs control-untransfected (no siRNA). # p<0.05 vs. untreated scramble siRNA-transfected cells. $ p<0.05 vs. CCN2(IV)-treated scramble siRNA-transfected cells. & p<0.05 vs. CCN2(IV)-treated untransfected cells.

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