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Article

Connective tissue growth factor is a new ligand of epidermal growth factor receptor

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Chronic kidney disease is reaching epidemic proportions worldwide and there is no effective treatment. Connective tissue growth factor (CCN2) has been suggested as a risk biomarker and a potential therapeutic target for renal diseases, but its specific receptor has not been identified. Epidermal growth factor receptor (EGFR) participates in kidney damage, but whether CCN2 activates the EGFR pathway is unknown. Here, we show that CCN2 is a novel EGFR ligand. CCN2 binding to EGFR extracellular domain was demonstrated by surface plasmon resonance. CCN2 contains four distinct structural modules. The carboxyl-terminal module (CCN2(IV)) showed a clear interaction with soluble EGFR, suggesting that EGFR-binding site is located in this module. Injection of CCN2(IV) in mice increased EGFR phosphorylation in the kidney, mainly in tubular epithelial cells. EGFR kinase inhibition decreased CCN2(IV)-induced renal changes (ERK activation and inflammation). Studies in cultured tubular epithelial cells showed that CCN2(IV) binds to EGFR leading to ERK activation and proinflammatory factors overexpression. CCN2 interacts with the neurotrophin receptor TrkA, and EGFR/TrkA receptor crosstalk was found in response to CCN2(IV) stimulation. Moreover, endogenous CCN2 blockade inhibited TGF-β-induced EGFR activation. These findings indicate that CCN2 is a novel EGFR ligand that contributes to renal damage through EGFR signalling.

Keywords: CCN2, receptors, EGFR, TrkA, renal, inflammation

Introduction

Chronic kidney disease is a major health problem that has reached epidemic proportions and it may lead to end-stage renal disease or early cardiovascular death. Moreover, available clinical treatments only retard renal disease progression. Connective tissue growth factor (CCN2/CTGF), a member of the CCN (Cyr61/CCN2/Nov) family, is over-expressed in many human renal pathologies (Perbal, 2004; De Winter et al., 2008). Experimental studies have shown that CCN2 inhibition slows disease progression in diabetic nephropathy, unilateral ureteral obstruction, and nephrectomized TGF-β1 transgenic mice (Yokoi et al., 2004; Okada et al., 2005; Guha et al., 2007; Phanish et al., 2010) suggesting that therapeutic approaches that selectively block CCN2 activity could be beneficial for renal disease treatment.

CCN2 has to be considered a matricellular protein rather than a conventional growth factor. This protein, as other CNN members, contains four distinct structural modules that can be cleaved by proteases: an amino-terminal insulin-like growth-factor-binding domain, a cysteine-rich domain, a thrombospondin type 1 repeat, and a carboxyl-terminal cystine-knot domain (Rachfal and Brigstock, 2005; Leask and Abraham, 2006; De Winter et al., 2008; Chen and Lau, 2010). CCN2 and its degradation fragments have been detected in biological fluids and have been proposed as risk biomarkers in several nephropathies (Riser et al., 2003; Tam et al., 2009; Slagman et al., 2011). Among these degradation fragments, the 11 kDa carboxyl-terminal module (namely here CCN2(IV)) has received special interest. In cultured cells, this fragment regulates cell migration and proliferation, increases chemokines and extracellular matrix production, and has been involved in renal inflammation (Liu et al., 2006; De Winter et al., 2008; Sanchez-Lopez et al., 2009; Markiewicz et al., 2011). Although several studies have investigated the intracellular mechanisms activated by CCN2 and its fragments, the identification of a specific receptor for CCN2 remains elusive.

The epidermal growth factor receptor (EGFR) is the founding member of the ErbB receptor tyrosine kinase family. EGFR signalling controls key cellular programmes, including survival, proliferation, differentiation, and locomotion, both during development and postnatally. The EGFR is over-expressed, dysregulated, or mutated in many epithelial malignancies, participating in human cancer including lung, colon, breast, ovary, and gliomas (Sibilia et al., 2007; Bronte

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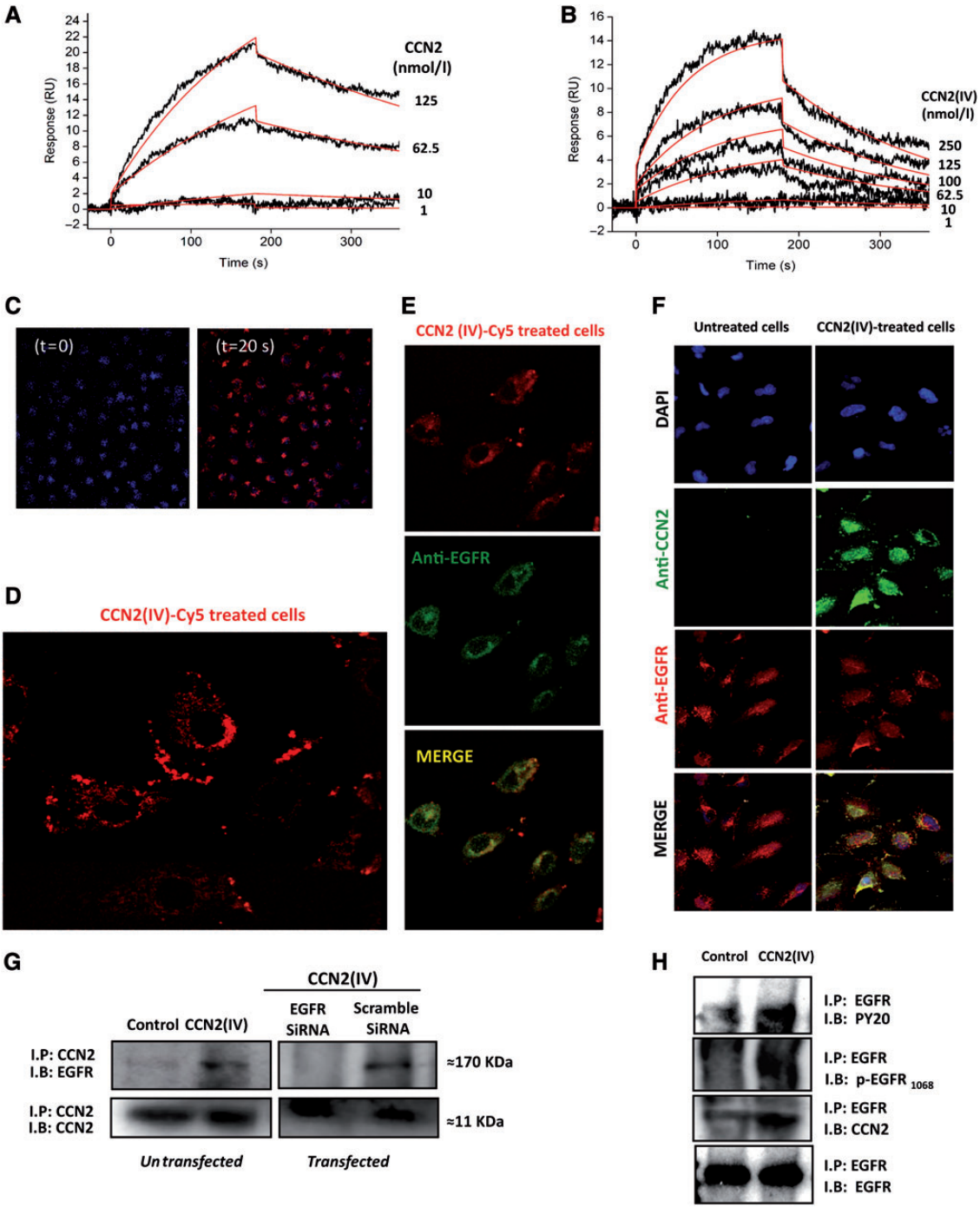

Figure 1 CCN2 binds to EGFR. Surface plasmon resonance interaction analysis of full-length CCN2 (**A**) and the carboxyl-terminal CCN2 fragment (CCN2(IV)) (**B**) with immobilized EGFR extracellular domain (sEGFR) was performed using Biacore 3000. Increasing concentrations of ligands (1–250 nmol/L) were injected over a surface with a density of 500 response units (RU) of immobilized sEGFR. The response in RU was recorded as a function of time. An overlay plot is shown of all sensorgrams after subtraction of their respective control sensorgrams. Binding parameters, calculated by applying the nonlinear curve-fitting software package BIAevaluation 3.2 (BIAcore, Inc.) to all sensorgrams simultaneously using a single-site model with drifting baseline, indicate that CCN2(IV)/EGFR interaction occurs with Kd = 126 ± 2 nmol/L. (**C** and **D**) CCN2(IV) interacts with EGFR in human tubular epithelial cells. Fluorescent labelled CCN2(IV)-Cy5 (100 ng/ml) was added to HK2 cells and live confocal microscopy images were taken once every 1.3 sec for a period of 2 min (time 0 and 20 sec, **C**; time 2 min, **D**). Nuclei were stained with DAPI (blue). CCN2–EGFR interaction was evaluated by immunocytochemistry. Serum-starved HK2 cells were stimulated or not with 100 ng/ml CCN2(IV)-Cy5 (**E**) or CCN2(IV) (**F**) for 10 min, and fixed by cross-linking. (**E**) CCN2(IV)-Cy5-treated cells presented a red membrane immunostaining, while EGFR was immunodetected by an secondary AlexaFluor®488 labelled antibody (green). EGFR/CCN2(IV) colocalization was found (yellow staining, merge). (**F**) EGFR and CCN2 were detected using specific primary antibodies followed by their corresponding secondary AlexaFluor®633/488 antibodies (red/green, respectively). EGFR/CCN2 colocalization was found only in CCN2(IV)-treated cells (yellow staining), but not in control ones. Figures show a

et al., 2011). Besides tumour biology, EGFR family members are implicated in the development of end organ damage in hypertension (Hao et al., 2004) and atherosclerosis (Dreux et al., 2006). In the kidney, EGFR signalling is critically involved in renal electrolyte homeostasis (Melenhorst et al., 2008), and EGFR blockade, by genetic or pharmacological approaches, ameliorates experimental renal disease progression (Terzi et al., 2000; Lautrette et al., 2005; Flamant et al., 2012; Liu et al., 2012). Our aim was to investigate the capacity of CCN2 and its carboxyl-terminal fragment to interact with and activate EGFR, and whether the activation of EGFR signalling is involved in CCN2-induced responses in the kidney.

Results

CCN2 binds to EGFR via the carboxyl-terminal module

Surface plasmon resonance analysis was used to assess the ability of CCN2 to bind the extracellular domain of EGFR. CCN2 bound to immobilized EGFR on a BIAcore sensor chip (Figure 1A). The full-length CCN2 protein contains four distinct structural modules (De Winter et al., 2008). Binding experiments using the carboxyl-terminal degradation fragment CCN2(IV) as a ligand showed a clear interaction with EGFR (Figure 1B), suggesting that the EGFR-binding site is present in the carboxyl-terminal module.

To investigate whether CCN2 directly interacts with EGFR in cells, we performed studies in cultured human tubular epithelial cells (HK2 cell line). First, live-cell imaging by confocal time-lapse microscopy was performed to visualize CCN2(IV) binding to the cell. After adding labelled CCN2(IV)-Cy5 to cells, the immunofluorescent signal was rapidly located at the cell membrane, indicating CCN2(IV) cellular binding (Figure 1C and D). The potential CCN2–EGFR interaction was further demonstrated by immunocytochemistry and immunoprecipitation (IP) experiments, using a cross-linking procedure to fix the proteins anchored to the cell surface. EGFR is expressed in untreated HK2 cells. Moreover, CCN2(IV)-treated cells showed a clear cellular binding that colocalized with EGFR immunostaining (Figure 1E and F). IP studies showed that in CCN2(IV)-treated cells, but not in untreated ones, CCN2–EGFR complexes were formed (Figure 1G and H). One of the earliest steps of EGFR activation is its auto-phosphorylation on tyrosine (Y) residues (Sweeney and Carraway, 2000). In CCN2(IV)-treated cells, complexes containing tyrosine-phosphorylated proteins, including Y1068 on EGFR, were found (Figure 1H). Transfection with a small interfering RNA molecule (siRNA) targeting EGFR, but not with a nonspecific scramble siRNA, abolished the CCN2–EGFR complex formation, showing the specificity of this interaction (Figure 1G). These data demonstrate that in cultured tubular epithelial cells, stimulation with CCN2(IV) led to CCN2–EGFR complex formation.

CCN2 induces EGFR phosphorylation in cultured tubular epithelial cells

In cultured human tubular epithelial cells, CCN2(IV) increased EGFR phosphorylation on Y1068 and Y1173 (Figure 2A). In murine tubular epithelial cells, CCN2 (IV)-induced EGFR activation was dose- and time-dependent, starting as early as 5 min and peaking after 15 min with a maximal response at 50 ng/ml (Figure 2B and C). EGFR specific activation was demonstrated by pharmacological inhibition using two different EGFR kinase inhibitors, erlotinib and AG1478 (Figure 2D), and EGFR gene silencing (Figure 2E). CCN2(IV) also increased EGFR phosphorylation in other cell types, including murine fibroblasts and human mesangial cells (Figure 2F and G). Moreover, the full-length CCN2 protein also induced EGFR phosphorylation (Figure 2H), showing a similar response as obtained with the carboxyl-terminal fragment CCN2(IV). For this reason, only CCN2(IV) was used in the following experiments. Our data demonstrate that both CCN2 and its carboxyl-terminal fragment bind and activate EGFR signalling in cells.

CCN2(IV) induces EGFR phosphorylation in the kidney

Next, we investigated whether CCN2 could activate EGFR signalling in the kidney. Renal levels of phosphorylated EGFR protein were elevated in CCN2(IV)-injected mice compared with control mice (Figure 3A). In the kidney, EGFR is mainly expressed in tubular cells (Melenhorst et al., 2008). Immunohistochemistry and immunofluorescence using antibodies that recognized phosphorylated EGFR on Y1173 and Y1068, respectively, revealed that CCN2(IV) activated EGFR in tubular cells *in vivo* (Figure 3B, C, and E). Treating the CCN2(IV)-injected mice with erlotinib, a small molecule tyrosine kinase inhibitor that targets the receptor catalytic domain of EGFR, diminished renal phosphorylated EGFR levels to control levels (Figure 3B, C, and E).

EGFR activation by CCN2(IV) is linked to ERK signalling

Several auto-phosphorylation sites have been identified in the carboxyl-terminal region of EGFR that varies dependent on the ligand and are linked to different downstream signalling systems (Sweeney and Carraway, 2000). EGFR phosphorylation on Y1068 and Y1173 is involved in ERK signalling (Rojas et al., 1996; Pourazar et al., 2008). Both tyrosines were phosphorylated in kidneys of CCN2(IV)-injected mice (Figure 3) and in cultured tubular epithelial cells exposed to CCN2(IV) (Figure 2A). CCN2(IV)-injected mice also presented elevated renal levels of phosphorylated ERK1/2 compared with controls, which were inhibited by erlotinib (Figure 3D). EGFR activation by CCN2 was also linked to ERK signalling in cultured tubular epithelial cells. Blockade of EGFR, by kinase inhibition or gene silencing, diminished ERK phosphorylation levels in CCN2(IV)-treated cells to levels similar to their corresponding controls (Figure 2D and E).

CCN2(IV) via EGFR activation regulates renal inflammatory response in vivo and in vitro

We further investigated the *in vivo* effect of EGFR blockade on CCN2-induced renal damage. Treatment of CCN2(IV)-injected mice with erlotinib diminished the presence of infiltrating monocytes/macrophages ($F4/80^+$ cells) and T lymphocytes ($CD3^+$

representative experiment of four with similar results. (**G**) CCN2–EGFR complexes were found by coprecipitation experiments. Cell lysates were immunoprecipitated with anti-CCN2, followed by SDS–PAGE and western blotting (IB) using an anti-EGFR antibody. In some points, cells were transfected with an EGFR siRNA or its corresponding scramble siRNA. In CCN2(IV)-treated cells, the 170 kDa band corresponding to EGFR molecular weight was found, while it disappeared in EGFR silenced cells, showing the formation of CCN2–EGFR complexes. The IB with anti-CCN2 antibody was used as loading control. (**H**) IP with anti-EGFR antibody followed by IB with several antibodies: anti-phosphorylated tyrosine (PY20), anti-phosphorylated EGFR ($p\text{-}EGFR_{1068}$), anti-CCN2, and anti-EGFR (used as loading control). In CCN2(IV)-treated cells, bands for PY20, $p\text{-}EGFR_{1068}$ and CCN2 were detected, confirming CCN2–EGFR complex formation. Figures show a representative IP experiment of five with similar results.

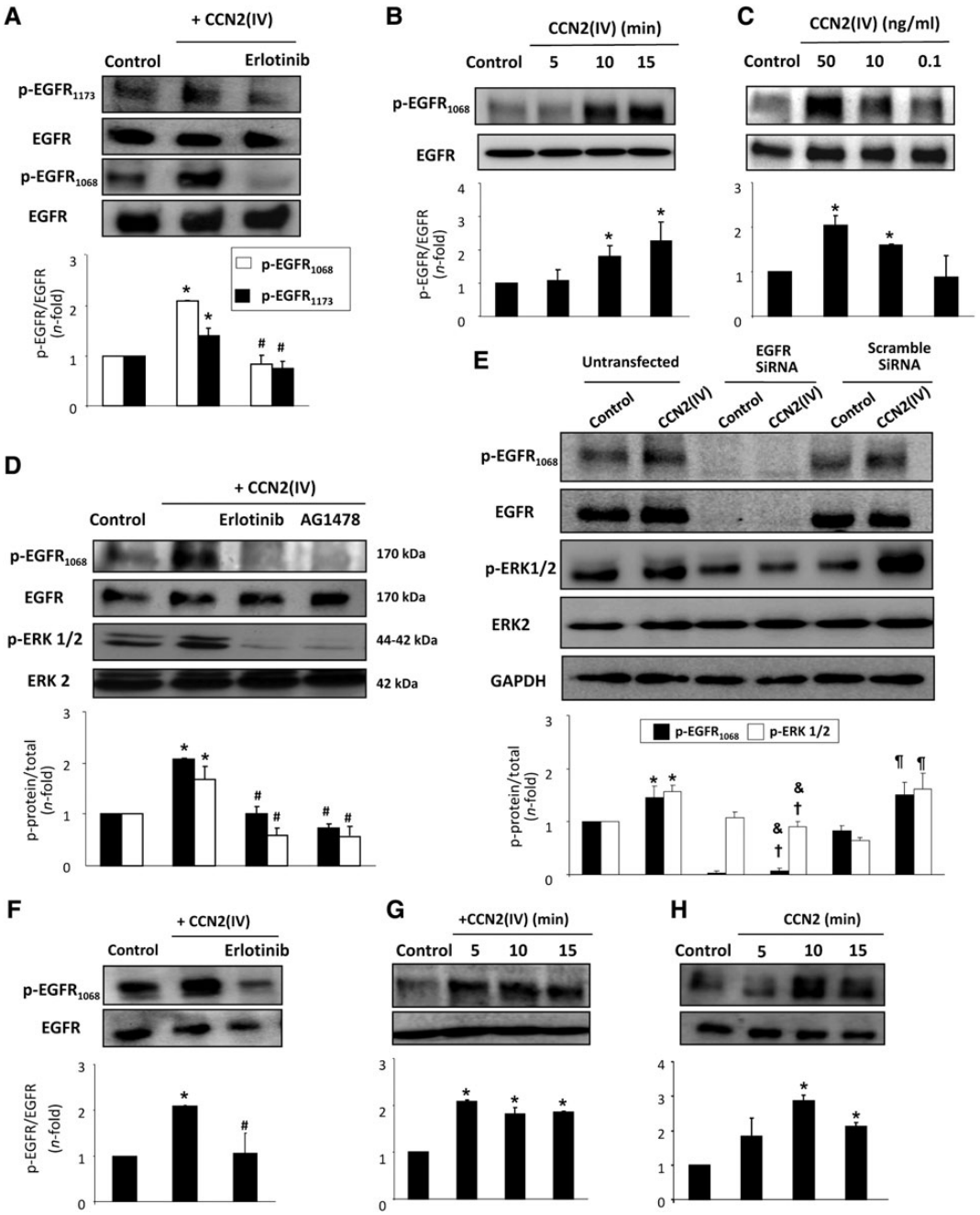

Figure 2 CCN2 activates EGFR signalling in renal cells. EGFR activation was evaluated using antibodies against phosphorylated EGFR on Y1068 (p-$EGFR_{1068}$) or Y1173 (p-$EGFR_{1173}$), both implicated in ERK activation. CCN2(IV) increased EGFR phosphorylation in human (**A**) and murine (**B**–**D**) tubular cells. (**A**) Human tubular epithelial cells (HK2 cells) were treated with 50 ng/ml CCN2(IV) for 15 min. Some cells were pre-incubated with erlotinib (10 μmol/L). Murine tubular epithelial cells were treated with 10 ng/ml CCN2(IV) for increasing time periods (**B**) or with several concentrations of CCN2(IV) (range 50–0.1 ng/ml) for 15 min (**C**). (**D**) Cells were pre-incubated for 1 h with erlotinib (10 μmol/L) or AG1478 (100 nmol/L) before the stimulation with 10 ng/ml CCN2(IV) for 15 min. (**E**) CCN2(IV) induces EGFR phosphorylation linked to ERK activation in tubular epithelial cells. HK2 cells were incubated with transfection reagent alone (untransfected) or transfected with EGFR siRNA or scramble siRNA, and then treated or not with CCN2(IV). ERK activity was determined by levels of phosphorylated ERK1/2. Total EGFR, ERK, and GAPDH levels were used as loading/silencing controls. Data of phosphorylated protein vs. total protein levels are expressed as mean ± SEM of 8 independent western blot experiments. $^{*}P < 0.05$ vs. control-untransfected. $^{¶}P < 0.05$ 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. Renal fibroblasts (**F**) and human mesangial cells (**G**) were treated with 10 ng/ml CCN2(IV) for 15 min and 50 ng/ml CCN2(IV) for increasing time periods (5, 10, and 15 min), respectively. (**H**) Full-length CCN2 activates the EGFR pathway. Murine tubular epithelial cells were stimulated with 34 ng/ml CCN2 (full-length recombinant protein) for increasing time periods. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV) alone. Figures (except **E**) show a representative western blot experiment and data are expressed as mean ± SEM of 5–8 independent experiments.

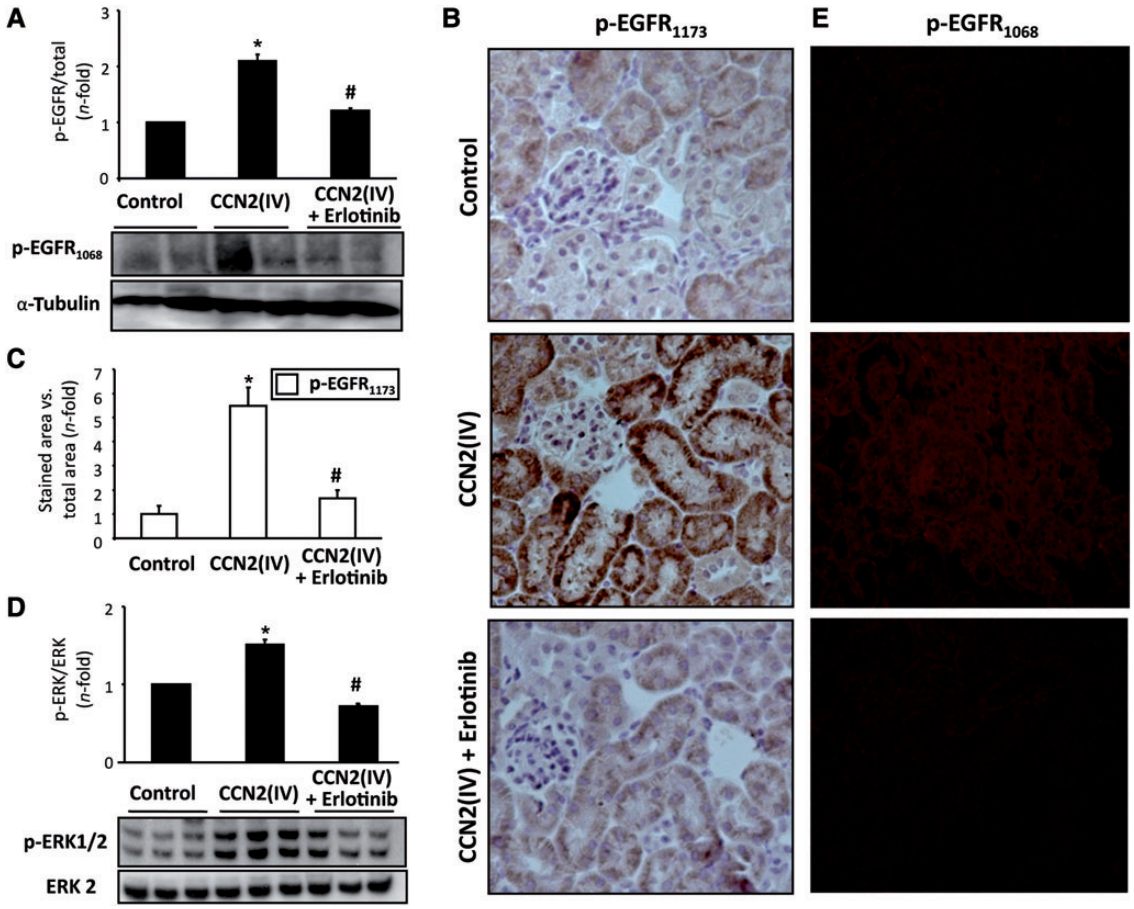

Figure 3 CCN2 induces EGFR phosphorylation in the kidney. (**A**) C57BL/6 mice were i.p. injected with 2.5 ng/g of body weight of recombinant CCN2(IV) or vehicle (saline) and sacrificed 24 h later. Some animals were treated with erlotinib (40 mg/kg per day) or its vehicle (control group), starting 24 h before CCN2(IV) injection. EGFR activation was determined in total renal extracts by western blot analysis. Figure shows two representative mice from each group and data are expressed as mean ± SEM of 8–10 mice per group. The localization of activated EGFR was determined by immunohistochemistry using antibody against p-EGFR$_{1173}$ (**B**) and by immunofluorescence using antibody against p-EGFR$_{1068}$ (**E**), which showed increased p-EGFR immunostaining mainly in tubular epithelial cells. (**C**) The pEGFR$_{1173}$ immunostaining in **B** was quantified and expressed as mean ± SEM of 8–10 animals per group. (**D**) ERK activation is a downstream mechanism of CCN2/EGFR signalling in the kidney. Figure shows a representative experiment and data of p-ERK levels are expressed as mean ± SEM of 8–10 mice per group. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV).

cells) in the kidney to levels similar to control mice (Figure 4A and B). EGFR inhibition also down-regulated renal gene expression and protein levels of several proinflammatory factors (CCL-2 and IL-6) to control levels (Figure 4C and D).

In cultured murine tubular epithelial cells, CCN2(IV) regulates some proinflammatory factors (Sanchez-Lopez et al., 2009). The blockade of EGFR by erlotinib or AG1478 diminished CCN2(IV)-induced gene overexpression and protein release of CCL-2 and IL-6 to control levels (Figure 4E and F). Similar inhibitory effect was found by EGFR gene silencing (Figure 4G). These data link EGFR activation by CCN2(IV) with the up-regulation of proinflammatory factors in tubular epithelial cells and the inflammatory response observed in the kidney.

ADAMs are not involved in CCN2-mediated EGFR-signalling activation

Besides direct activation of EGFR by ligand binding, several factors can indirectly activate EGFR by a process termed 'transactivation'. EGFR transactivation is regulated by ADAMs, disintegrins, and matrix metalloproteases (MMPs) that mediate EGFR ligand shedding (Ohtsu et al., 2006). In renal cells, ADAM-17 regulates EGFR transactivation (Lautrette et al., 2005; Wolf, 2005). We have observed that a pan-specific inhibitor of MMPs, GM6001, did not modify CCN2(IV)-induced EGFR phosphorylation (Figure 5A). Moreover, the pharmacological inhibition of ADAM-17 using TAPI-2 or ADAM-17 gene silencing did not modify CCN2(IV)-induced EGFR phosphorylation (Figure 5A and B). These data clearly demonstrate that ADAMs are not involved in CCN2-mediated EGFR-signalling activation, and support our findings that CCN2 directly interacts with EGFR.

Role of integrins in CCN2-induced EGFR activation

Integrins are heterodimeric receptors for cell-surface adhesion molecules and extracellular matrix proteins, which are composed of two subunits, α and β. Each αβ combination has specific signalling properties (Juliano, 2002). To date, eighteen α and eight β subunits have been identified, which form at least 24 different αβ integrins (Humphries et al., 2006). Integrin-binding sites are present in CCN2 and mediate several effects (Chen et al., 2004; Gao and Brigstock, 2005). We first tested the involvement of integrins in EGFR activation

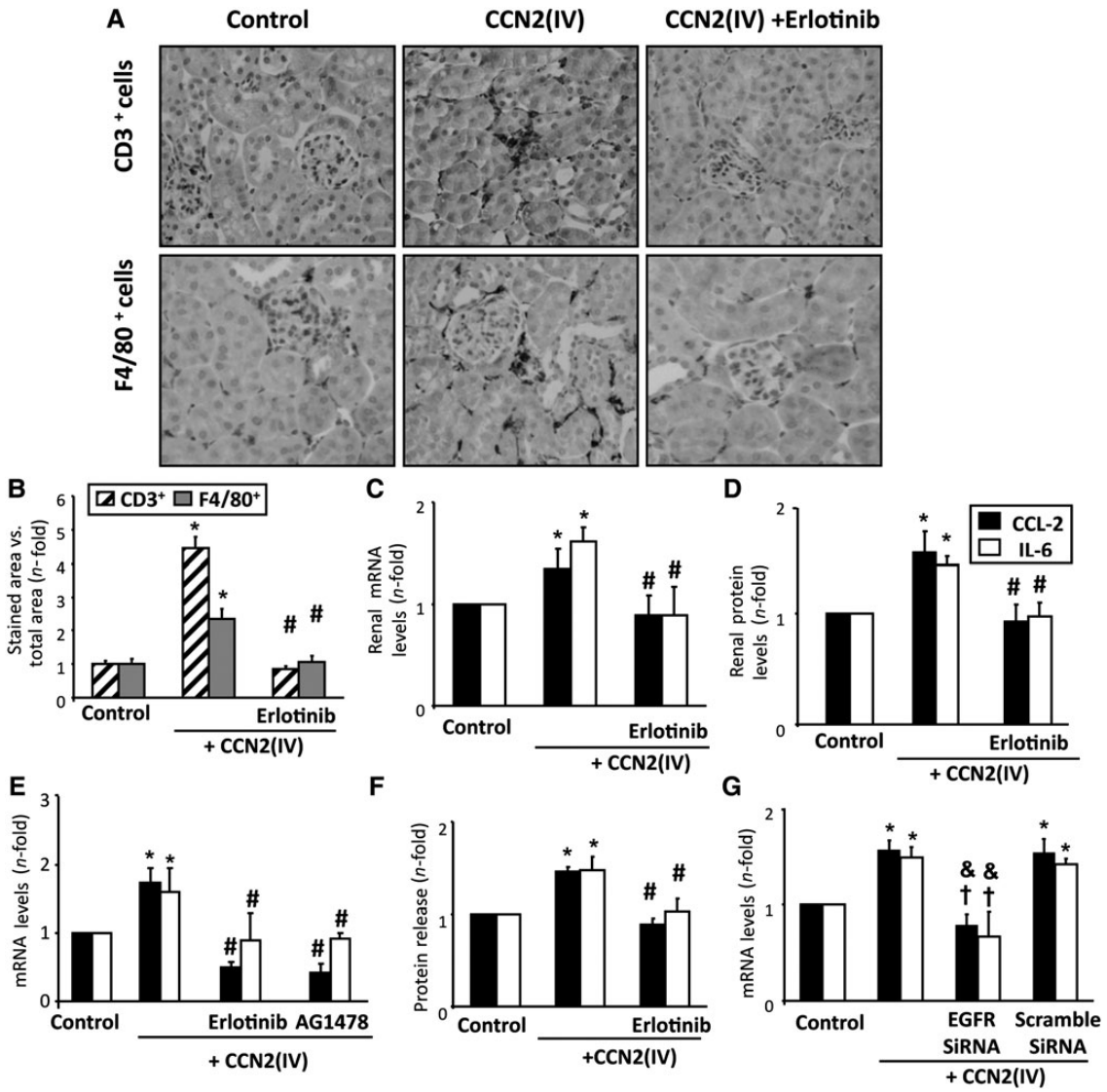

Figure 4 EGFR kinase inhibition decreases CCN2(IV)-induced renal inflammatory cell infiltration. In paraffin-embedded kidney sections, immunohistochemistry using anti-F4/80 and anti-CD3 was performed to characterize monocyte/macrophages and T lymphocytes, respectively (representative sections from each group, **A**; staining quantification, **B**). Magnification 200×. Erlotinib inhibits CCN2(IV)-induced up-regulation of renal inflammatory molecules. CCL-2 and IL-6 gene expression levels were determined by real-time PCR (**C**) and protein levels by ELISA (**D**) in total renal extracts from different animal groups. Data are expressed as mean ± SEM of 8–10 animals per group. $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV). (**E**–**G**) CCN2(IV) increases proinflammatory factors via EGFR activation in murine tubular epithelial cells. Cells were pre-incubated for 1 h with erlotinib (10 μmol/L) or AG1478 (100 nmol/L) before the stimulation with 10 ng/ml CCN2(IV) for 6 h (gene studies; **E**) or 24 h (protein studies, cell-conditioned medium; **F**). (**G**) EGFR gene silencing inhibits upregulation of proinflammatory molecules caused by CCN2(IV) in human tubular epithelial cells. HK2 cells were transfected or not with an EGFR or scramble siRNA before the stimulation with 50 ng/ml CCN2(IV) for 6 h (gene studies). Data are expressed as mean ± SEM of 6 (**E**), 5 (**F**), and 4 (**G**) independent experiments. $^{*}P < 0.05$ vs. control-untransfected. $^{\#}P < 0.05$ vs. CCN2(IV). $^{\dagger}P < 0.05$ vs. CCN2(IV)-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. CCN2(IV)-treated untransfected cells.

by CCN2(IV) using arginine-glycine-aspartic acid (RGD) peptides. RGD was originally identified as the sequence in fibronectin that is a recognition site for α5β1 integrin, but it also recognizes α3β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, and α2bβ3 integrins (Plow et al., 2000). Pre-incubation of HK2 cells with cyclic RGD peptide (GRGDSP), but not with control peptide (GRGDESP), reduced CCN2(IV)-induced EGFR activation (Figure 6A), suggesting that integrins with RGD recognition specificity may be involved in CCN2 responses.

The specific integrins involved in CCN2 actions are cell-dependent. In hepatic stellate cells, αVβ3 integrin interacts with CCN2(IV) (Gao and Brigstock, 2004), while in pancreatic stellate cells it is α5β1 (Gao and Brigstock, 2005). Therefore, we tested the involvement of these two integrins in tubular epithelial cells. Pre-incubation of HK2 cells with a neutralizing antibody against αVβ3 integrin inhibited CCN2(IV)-induced EGFR phosphorylation, while a control IgG or a α5β1-neutralizing antibody had no effect (Figure 6B). Using siRNA against β3 or αV integrins showed

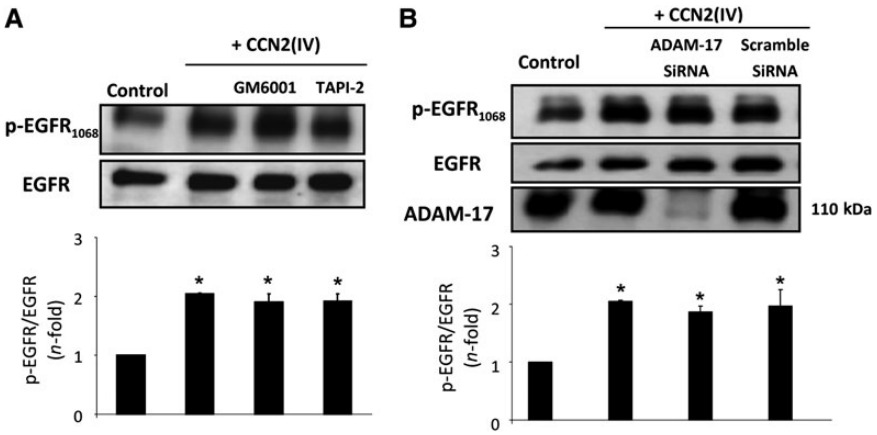

Figure 5 Pharmacological inhibition of MMPs or ADAM-17 and gene silencing of ADAM-17 do not modify CCN2(IV)-induced EGFR phosphorylation in human tubular epithelial cells. (**A**) HK2 cells were pre-incubated for 1 h with the pan-specific MMPs inhibitor GM6001 (1 μmol/L) or the ADAM-17-specific inhibitor TAPI-2 (20 μmol/L) before the stimulation with 50 ng/ml CCN2(IV) for 15 min. Values are mean ± SEM from at least 6 independent experiments. *$P < 0.05$ vs. control. (**B**) HK2 cells were transfected with ADAM-17 siRNA or scramble siRNA, and then treated with 50 ng/ml CCN2(IV) for 15 min. Values are mean ± SEM from at least 4 independent experiments. *$P < 0.05$ vs. control-untransfected.

similar inhibitory effects, while a scrambled siRNA had no effect (Figure 6C and D). These data clearly show that αVβ3 integrin mediates CCN2(IV)-induced EGFR activation.

Next, we evaluated whether CCN2(IV) could directly bind to αVβ3 integrin by IP experiments. In CCN2(IV)-treated HK2 cells, the formation of CCN2-β3 and CCN2-αV complexes was found (Figure 6E). Moreover, EGFR gene silencing did not modify the CCN2(IV) binding to αV or β3 integrin subunits (Figure 6E), demonstrating the direct binding of CCN2(IV) to αVβ3 integrin, even in the absence of EGFR. Interestingly, pre-incubation of cells with the neutralizing antibody against αVβ3 integrin did not modify the CCN2–EGFR complex formation, assessed by EGFR co-IP upon CCN2(IV) treatment (Figure 6F). These data showed that αVβ3 integrin directly binds to CCN2(IV), but is not necessary for the binding of CCN2(IV) to EGFR and the subsequent complex formation.

Potential crosstalk between EGFR and TrkA in response to CCN2(IV) stimulation in cultured tubular epithelial cells

In mesangial cells, CCN2 stimulated tyrosine phosphorylation of proteins at 75–80 and 140–180 kDa within 10 min, and previous studies have identified the neurotrophin receptor TrkA (molecular weight ~140 kDa) as a potential CCN2 receptor (Wahab et al., 2005), also in other cell types, such as cardiomyocytes (Wang et al., 2010). Therefore, the role of TrkA in CCN2-induced responses in tubular epithelial cells was evaluated. Western blot was performed using an antibody that recognizes TrkA phosphorylated on Y490, previously related to CCN2 responses in mesangial cells (Wahab et al., 2005). We found that CCN2(IV) increased TrkA phosphorylation levels in HK2 cells, which was abolished in TrkA-silenced cells (Figure 7A), showing the specificity of this CCN2(IV) response.

Next, we further evaluated the potential interrelation between EGFR and TrkA in HK2 cells. Gene silencing of TrkA diminished CCN2(IV)-induced EGFR phosphorylation (Figure 7A). Moreover, pharmacological inhibition of TrkA using K252a also blocked CCN2(IV)-mediated EGFR activation (Figure 7B). On the other hand, EGFR gene silencing inhibited TrkA phosphorylation induced by CCN2(IV) (Figure 7C). These data indicate an EGFR/TrkA receptor crosstalk.

CCN2 is a downstream mediator of TGF-β-induced EGFR activation

CCN2 is a downstream mediator of TGF-β-induced profibrotic responses (Ruiz-Ortega et al., 2007). In HK2 cells, blockade of endogenous CCN2 production by specific CCN2 gene silencing markedly diminished TGF-β-induced EGFR phosphorylation after 24 h of TGF-β incubation, compared with scramble siRNA controls (Figure 8). Our results confirm and extend previous data, showing that CCN2 is a downstream mediator of TGF-β-induced responses, including EGFR signalling.

Discussion

By surface plasmon resonance, we have detected direct binding of CCN2 to the immobilized extracellular fraction of EGFR. Interestingly, both full-length CCN2 and CCN2(IV) bound to EGFR and increased EGFR phosphorylation in cultured renal cells, suggesting that the EGFR-binding site is present in the carboxyl-terminal module. Our *in vivo* studies show that CCN2(IV) activates EGFR/ERK pathway in the kidney, mainly in tubular epithelial cells. Our *in vitro* studies in these cells demonstrate that CCN2(IV) rapidly binds to the cellular membrane and leads to CCN2–EGFR complex formation, increases EGFR phosphorylation, and activates downstream signalling mechanisms. Seven ligands for EGFR have been identified so far: EGF, TGF-α, heparin binding EGF-like growth factor, amphiregulin, betacellulin, epigen, and epiregulin (Dreux et al., 2006). Data presenting here extend this list, suggesting that CCN2 is another ligand for EGFR.

Ligand binding to EGFR induces a conformational change leading to the formation of receptor homo- or heterodimers and subsequent activation of the intrinsic tyrosine kinase domain by phosphorylation of specific tyrosine residues within the cytoplasmic tail of the receptor (Sweeney and Carraway, 2000). Phosphorylation of different tyrosine residues occurs upon binding of different ligands to the same EGFR, leading to a variety of downstream signal transduction pathways that can be selectively activated (Sweeney and Carraway, 2000). Our *in vivo* data clearly demonstrated that CCN2(IV) administration activated renal EGFR, as shown by increased EGFR phosphorylation, mainly in tubular epithelial cells. In these cells *in vitro* and *in*

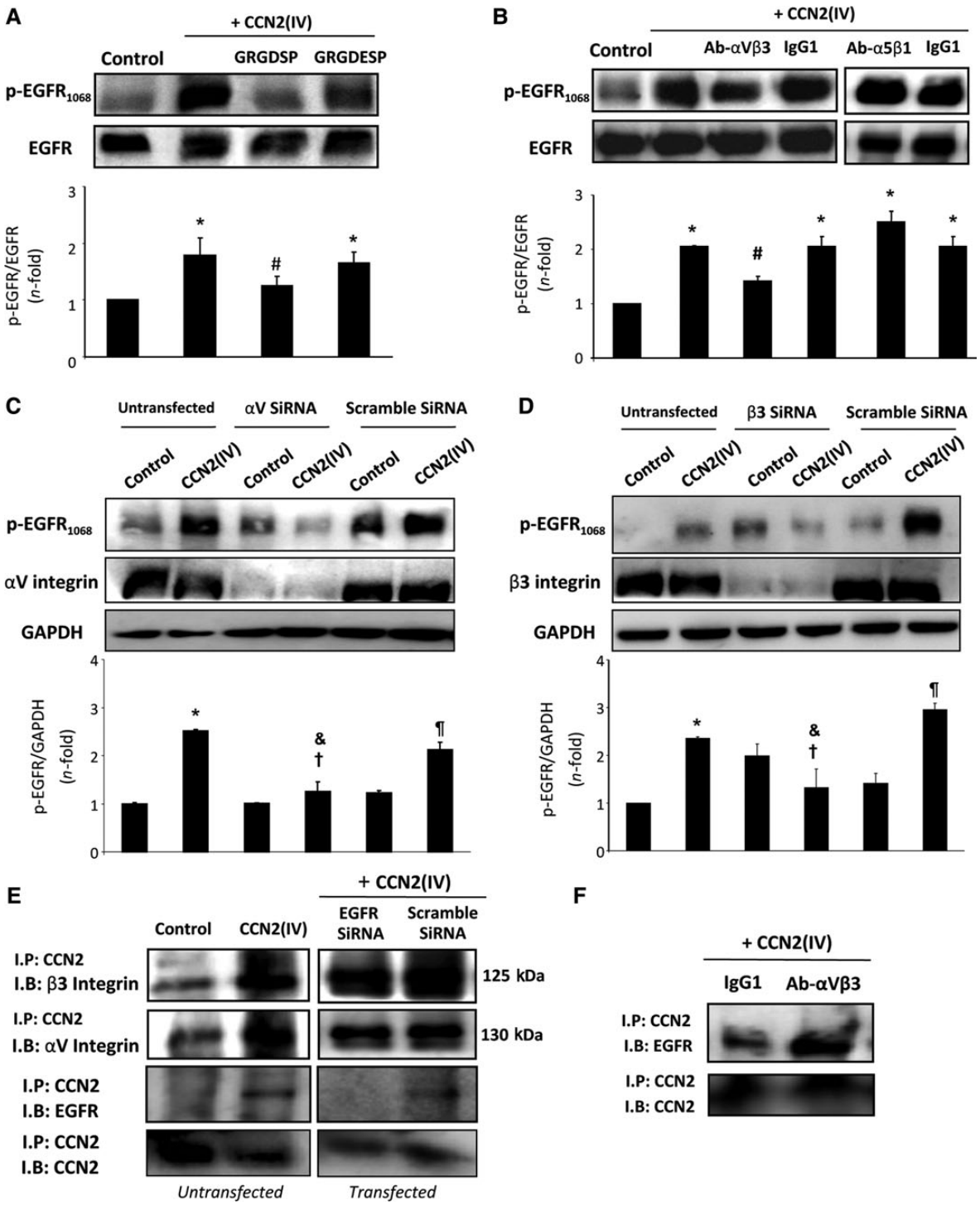

Figure 6 Role of integrins in CCN2(IV)-induced EGFR activation in cultured human tubular epithelial cells. HK2 cells were pre-incubated with 0.2 mmol/L RGDSP or its control peptide RGDESP (**A**) or with 5 μg/ml neutralizing antibodies against αVβ3 integrin, α5β1 integrin, or their corresponding IgG1 control (**B**). $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. CCN2(IV). HK2 cells were non-transfected or transfected with αV (**C**), β3 (**D**), or scramble siRNA, and then treated or not with 50 ng/ml CCN2(IV) for 15 min. Figures show a representative western blot and data are expressed as mean ± SEM of 8 independent experiments. αV or β3 integrin was used as silencing control and GAPDH as loading control. $^{*}P < 0.05$ vs. control-untransfected. $^{\P}P < 0.05$ vs. untreated scramble siRNA-transfected cells. $^{\dagger}P < 0.05$ vs. CCN2(IV)-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. CCN2(IV)-treated untransfected cells. (**E**) EGFR gene-silenced cells were stimulated with CCN2(IV) and cross-linked, and cell lysates were immunoprecipitated with anti-CCN2 antibody (IP) and analysed by western blot (IB) with antibodies against EGFR, CCN2, αV, or β3 integrins. The formation of αV/β3-CCN2(IV) complexes in the presence or absence of EGFR (blocked by gene silencing) was shown. (**F**) Cells were pre-incubated with a neutralizing αVβ3 integrin antibody or IgG control before CCN2(IV) stimulation. The αVβ3 integrin neutralization did not modify CCN2(IV)-EGFR complex formation. One representative experiment out of three with similar results was shown in **E** and **F**.

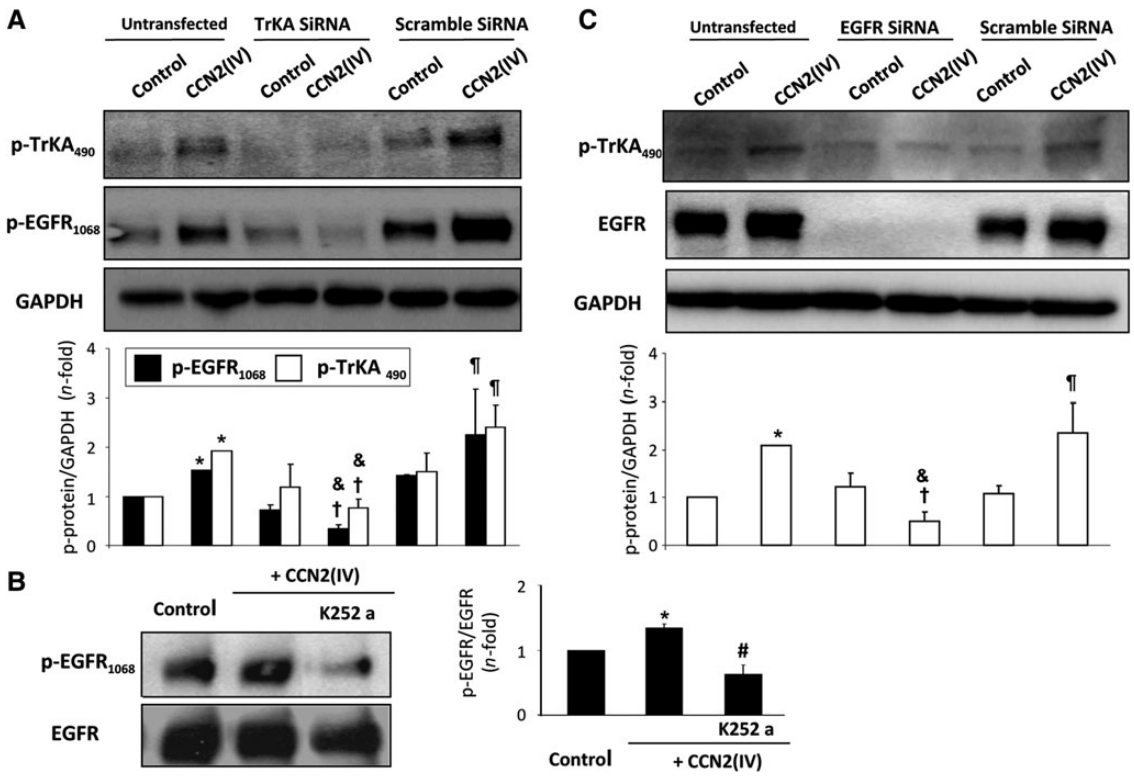

Figure 7 Blockade of TrkA by gene silencing or a specific TrkA receptor inhibitor inhibits CCN2(IV)-induced EGFR activation and EGFR gene silencing inhibits CCN2(IV)-induced TrkA activation. (**A** and **C**) HK2 cells were transfected with siRNA against TrkA (**A**) or EGFR (**C**) or its corresponding scramble. (**B**) HK2 cells were pre-incubated with 1×10^{-4} mmol/L K252a for 1 h. In all experiments, cells were treated or not with 50 ng/ml CCN2(IV) for 15 min. Activation of EGFR or TrkA was evaluated using specific p-EGFR$_{1068}$ or p-TrkA$_{490}$ antibodies. EGFR and pTrkA were used as silencing controls and GAPDH as loading control. Figures show a representative western blot experiment and data are expressed as mean ± SEM of 4 (**A**), 3 (**B**), and 4 (**C**) independent experiments. $^{*}P < 0.05$ vs. control-untransfected. $^{¶}P < 0.05$ vs. untreated scramble siRNA-transfected cells. $^{\dagger}P < 0.05$ vs. CCN2(IV)-treated scramble siRNA-transfected cells. $^{\&}P < 0.05$ vs. CCN2(IV)-treated untransfected cells.

vivo, CCN2(IV) induced EGFR phosphorylation on Y1068 and Y1173, which have been previously associated to MAPK cascade (Rojas et al., 1996; Sweeney and Carraway, 2000). In cultured tubular epithelial cells, EGFR blockade by gene silencing or kinase inhibition inhibited CCN2(IV)-induced ERK activation and upregulation of proinflammatory genes. *In vivo* treatment with erlotinib markedly diminished the number of inflammatory cells, the up-regulation of proinflammatory markers, and ERK activation in the kidneys of CCN2(IV)-injected mice. Our results suggest that CCN2(IV) directly binds to EGFR and activates its signalling pathway leading to the modulation of downstream mechanisms, such as ERK activation, and cellular responses, including renal inflammatory cell infiltration.

EGF ligands exist as inactive transmembrane precursors, requiring ADAM-mediated proteolytic cleavage of their ectodomain to be released as mature soluble ligands, whereby ADAMs regulate EGFR ligands availability (Melenhorst et al., 2008). Regarding CCN2, the full-length protein can be digested by proteases including MMPs. In particular, MMP-2 leads to the generation of a 11 kDa carboxyl-terminal fragment, which corresponds to CCN2(IV) (Hashimoto et al., 2002; De Winter et al., 2008; Tam et al., 2009). In the urine of patients with diabetic nephropathy, full-length CCN2 and CCN2(IV) were both found (Riser et al., 2003). However, the mechanisms involved in the regulation of CCN2 degradation in renal diseases are unknown and future studies are needed.

EGFR transactivation is mediated by ADAM-dependent EGFR ligand shedding by factors that bind G protein-coupled receptors (Ohtsu et al., 2006). Depending on the tissue, different ADAMs may be involved in EGFR ligand shedding. In this sense, Angiotensin II-induced EGFR transactivation in the kidney is regulated by ADAM-17 (Lautrette et al., 2005; Wolf, 2005; Flamant et al., 2012), while ADAM-12 mediates this process in the heart (Asakura et al., 2002). We have observed that the pharmacological inhibition of MMPs or ADAM-17 and gene silencing of ADAM-17 did not modify CCN2(IV)-induced EGFR phosphorylation in renal cells, demonstrating that MMPs are not involved in CCN2(IV)-mediated EGFR-signalling activation and confirming the direct interaction of CCN2(IV) with EGFR.

Several *in vitro* studies have shown that CCN2, through its binding to different domains, regulates different processes. The aminus terminal portion binds IGF-I and synergizes in the production of matrix proteins in renal cells (Kim et al., 1997; Wang et al., 2001; Lam et al., 2003). In *Xenopus* cells, CCN2, through the cysteine-rich domain, directly binds to TGF-β and acts as a cofactor that enhances TGF-β binding to its receptors and Smad-responsive promoter activation (Abreu et al., 2002). TSP-1 domains have been implicated in the binding to extracellular matrix proteins, integrins, heparan sulphate proteoglycans, low-density lipoprotein receptor-related protein, and vascular endothelial growth factor (Adams and Tucker, 2000; Segarini et al., 2001; Inoki et al., 2002; Leask and Abraham, 2006;

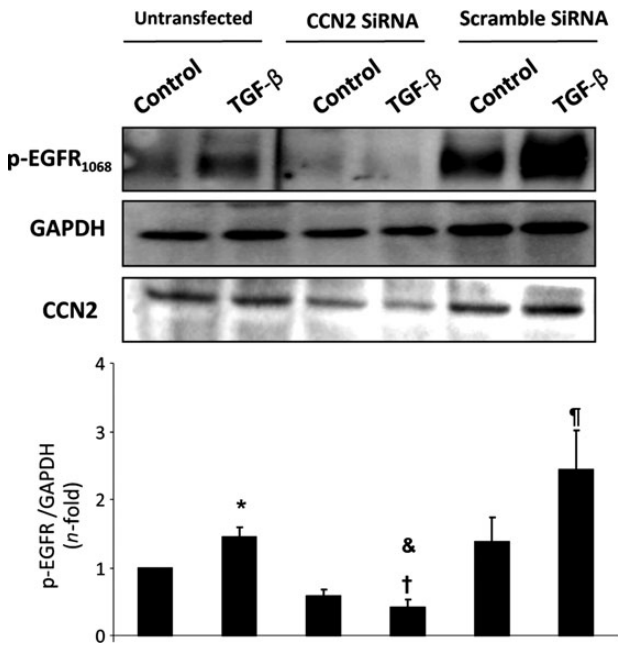

Figure 8 Blockade of endogenous CCN2 production diminishes TGF-β-induced EGFR activation. CCN2 gene silenced HK2 cells and control cells were stimulated with 1 ng/ml TGF-β for 24 h. Figure shows a representative experiment and data are expressed as mean ± SEM of 3 independent western blot. *$P < 0.05$ vs. control-untransfected. ¶$P < 0.05$ vs. untreated scramble siRNA-transfected cells. †$P < 0.05$ vs. TGF-β-treated scramble siRNA-transfected cells. &$P < 0.05$ vs. TGF-β-treated untransfected cells.

Chen and Lau, 2010). The carboxyl-terminal CCN2 cystine-knot module binds to integrins and exerts additional signalling capabilities, including regulation of fibrosis and inflammation (Leask and Abraham, 2006; Liu et al., 2006; De Winter et al., 2008). Our *in vitro* data show that this carboxyl-terminal fragment binds to EGFR leading to the regulation of proinflammatory factors. Our findings implicate integrins as key mediators of CCN2(IV)-induced EGFR activation using RGD peptides and provide evidence that integrin αVβ3 is involved in CCN2(IV)-induced EGFR activation based on results of the *in vitro* experiments utilizing neutralizing anti-integrin antibodies and siRNA. The ability of integrins to cooperate with receptor tyrosine kinases, including EGFR, to transduce proliferative signals and regulate cell survival and migration has been discussed previously (Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002). Integrins are able to form physical complexes with EGFR at the cell membrane and trigger ligand-independent phosphorylation of Y845, Y1068, Y1086, and Y1173 residues in the EGFR molecule (Moro et al., 1998). This integrin-dependent EGFR activation appears necessary for full EGFR-dependent transcriptional responses (Cabodi et al., 2004). Our data show that αVβ3 integrin binds to CCN2(IV) and is involved in EGFR-signalling transduction, but is not necessary for its binding to the EGFR and the formation of EGFR-CCN2(IV) complex.

TrkA is a member of the Trk family of cell membrane receptors (TrkA, TrkB, and TrkC). These receptors interact with neurotrophins and form homodimers or heterodimers with the low-affinity pan neurotrophin receptor, p75NTR. Neurotrophins, such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3 and 4, and their receptors, are important for the development, survival, and function of neurons (Allen and Dawbarn, 2006). The neurotrophin receptor TrkA has been proposed as a CCN2 receptor in mesangial cells (Wahab et al., 2005) and involved in diabetic nephropathy (Fragiadaki et al., 2012). In murine cardiomyocytes, CCN2 via TrkA induced profibrotic and proinflammatory effects (Wang et al., 2010). Interestingly, cardiomyocytes express additional CCN2 receptors that mediate proinflammatory actions, since CCN2-induced TNF-α and IL-6 mRNA upregulation occurred in the absence of TrkA (Wang et al., 2010). In tubular epithelial cells, we have found that CCN2(IV) activates TrkA signalling. Gene silencing and pharmacological inhibition of TrkA diminished EGFR phosphorylation, and EGFR gene silencing inhibited TrkA phosphorylation induced by CCN2(IV), demonstrating EGFR/TrkA crosstalk in response to CCN2(IV) stimulation. The similarity of phosphoproteomic profiles between TrkA and EGFR indicates a considerable overlap in downstream signallings originated in these tyrosine kinase receptors (Bradshaw et al., 2013). In monocytes, EGFR/TrkA crosstalk has been described in response to G protein-coupled receptors and linked to modulation of proinflammatory mediators (El Zein et al., 2010). The complexity of tyrosine kinase receptor signalling and interactions will require future studies.

The incidence of chronic kidney disease is increasing and current treatments only retard disease progression. Many studies using different strategies for blocking CCN2 activity have proven beneficial effects on experimental pathologies, including renal diseases (Leask and Abraham, 2006) However, these are far from being used in humans. CCN2 overexpression has been described in a wide variety of progressive human renal diseases and has been proposed as a risk biomarker (Riser et al., 2003; Tam et al., 2009; Slagman et al., 2011). We describe here that CCN2 (both full-length and the carboxyl-terminal fragment) interacts with and activates EGFR, leading to ERK activation and regulation of renal inflammation. CCN2 has been described as a downstream mediator of profibrotic factors (Hashimoto et al., 2002; Ruperez et al., 2003; Leask and Abraham, 2006). Our results showing that CCN2 gene silencing inhibited EGFR pathway activation in response to TGF-β support these findings and extend the importance of EGFR signalling in the fibrotic process. Experimental evidences suggest that EGFR inhibition may have therapeutic potential for kidney diseases (Lautrette et al., 2005; Flamant et al., 2012; Liu et al., 2012). Our findings indicate that CCN2 is a new ligand of the EGFR and identify this receptor as an important therapeutic target for renal diseases.

Materials and methods

Cell cultures

Human renal proximal tubular epithelial cells (HK2 cell line, ATCC CRL-2190) were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin transferrin selenium (ITS), and 36 ng/ml hydrocortisone in 5% CO_2 at 37°C. Murine renal cortical fibroblasts (TFB cell line) and murine proximal tubular epithelial cells [murine tubular-epithelial (MCT) cell line] originally obtained from Dr Eric Neilson (Vanderbilt University) were grown in RPMI with 10% FBS, 2 mmol glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. At 60%–70% of confluence, HK2 and TFB cells were growth-arrested in serum-free medium for 24 h

before the experiments, while MCT cells were maintained in 1% FBS.

Reagents

The full-length molecule of CCN2 (CCN2; Biovendor), TGF-β and CCN2(IV) (Preprotech), erlotinib (Vichem), tyrphostin AG1478 (Alomone Labs), K-252a and GM6001 (Calbiochem), TAPI-2 (Enzo Life Sciences), RGDs peptides (Bachem), and neutralizing. Neutralizing antibodies against integrin αVβ3, integrin α5β1, and IgG1 (Millipore) were used. DMSO, used as solvent of some reagents, had no effect on cell viability or gene expression.

Ligand–receptor interaction assays

Surface plasmon resonance interaction analysis was performed using Biacore 3000 (GE Healthcare). Data were collected using the highest collection rate. All experiments were carried out at 25°C using HBS-EP (10 mmol/L HEPES, 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005% P20, pH 7.4) as running buffer. CM5 sensor chip, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC), and ethanolamine HCl were obtained from GE Healthcare. EGFR extracellular domain (Genway) was immobilized on the surface of a CM5 sensor chip by the standard amino coupling procedure at a flow rate of 5 μl/min. The surface was activated for 7 min using a 0.05 mol/L NHS/0.2 mol/L EDC mixture. Then, 5 μg/ml EGFR in 10 mmol/L sodium acetate (pH 5.0) was injected for 13 min. Finally, residual activated groups on the surface were deactivated by a 7 min injection of 1 mol/L ethanolamine (pH 8.5). Immobilization density reached 500 RU. An additional flow cell was activated and deactivated and then used as a reference surface. For kinetic analysis, CCN2 and CCN2(IV) were diluted in HBS-EP (range 1–250 mmol/L). Concentration series and blank samples were injected for 3 min using a flow rate of 50 μl/min and the dissociation was monitored for 3 min. Data processing and kinetic analysis were performed using BiaEvaluation 4.1.1. (GE Healthcare). Data were double referenced using reference surface subtraction and blank correction. Processed data were globally fit to a simple 1:1 interaction model.

Live cell confocal microscopy

Cells were imaged using a Leica TCS SP5 confocal microscope. Fluorophore Cy-5-emitted fluorescence was monitored with a 550 ± 2 nm band pass or a 670 nm long pass filter and DAPI was excited using a DIODE laser. For video rate confocal, the images were captured (1 frame every 1.33 sec) at 400 Hz for a period of 2 min and digitalized using the LIF/LEICA program (LEICA microsystems). CCN2 fluorescence by Cy5 labelling was detected using Cy-5 fluorophore (1 nmol/μl; Amersham) following the manufacturer instructions.

Chemical cross-linking

Chemical cross-linking was carried out as previously described, using DTSSP (Pierce) (Ardura et al., 2010), before immunocytochemistry and co-IP experiments.

Fluorescence immunocytochemistry

Growth-arrested HK2 cells growing on glass coverslips were stimulated with CCN2(IV) or CCN2(IV)-Cy5. After chemical cross-linking, cells were fixed in 4% paraformaldehyde, washed, and blocked (PBS/10% BSA/4% serum, 1 h). Then, cells were incubated overnight with anti-EGFR (1:200 dilution; Santa Cruz Biotechnology) or anti-CCN2 (1:200 dilution; Sigma) in PBS with 1% BSA, followed by AlexaFluor®633-conjugated goat anti-mouse (red) or AlexaFluor®488-conjugated goat anti-rabbit (green) antibodies (1:300 dilution; Invitrogen), respectively. Nuclei were stained with 1 μg/ml DAPI as control for equal cell density. The absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40–88 (Sigma) and examined by using a Leica DM-IRB confocal microscope.

Co-IP assays

After chemical cross-linking, cells were lysed in 300–500 μl Triton–NP-40 lysis buffer [50 mmol/L Tris–HCl pH 8, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulphonylfluoride, 1% NP-40/IGEPAL, and a phosphatase-inhibitor cocktail (Set II, Calbiochem)], scraped off the dish, and incubated for 1 h at 4°C with shaking. Cell lysates were pre-cleared by incubating with 10 μl of protein A-agarose bead slurries (0.5 ml agarose/2 ml PBS) for 30 min at 4°C, and then centrifuged to wash away supernatants. Pre-cleared lysates were incubated with 2.5–5 μg antibody overnight at 4°C for IP experiment. The immune complexes were captured by the addition of protein A/G PLUS-agarose (20 μl) bead slurries for 1 h at 4°C. The agarose beads were collected by centrifugation, washed and subjected to SDS–PAGE, followed by western blot as described below (Ardura et al., 2010).

Western blot

Proteins were obtained from treated 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 inhibitors cocktail, 10 μl/ml PMSF, and 10 μl/ml orthovanadate). To determine protein content the BCA method was used. Cell (25 μg/lane) and kidney (100–150 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% defatted milk/0.05% Tween-20, and incubated overnight at 4°C with the following antibodies (dilution): p-EGFR$_{1068}$ (1:250; Calbiochem), p-EGFR$_{1173}$ (1:250; Cell Signalling), p-TrkA$_{490}$ (1:1000; Cell Signalling), ADAM-17 (1:1000; Abcam), CCN2 (1:1000; Sigma), EGFR (1:250), ERK 1/2 (1:200), integrin αV (1:200), integrin β3 (1:200), pERK1/2 (1:200), and anti-phosphotyrosine PY20 (1:250; Santa Cruz). Membranes were subsequently incubated with peroxidase-conjugated IgG secondary antibody and developed using an ECL chemiluminiscence kit (Amersham). Loading controls were done using an anti-GAPDH antibody (1:10000; Chemicon) or total protein levels in phosphorylation studies. Autoradiographs were scanned using the Gel Doc™ EZ imager and analysed with the Image Lab 3.0 software (BioRad).

Gene silencing

Gene silencing in cultured cells was performed using either a pre-designed siRNA corresponding to EGFR, ADAM-17, TrkA, CCN2 (Ambion), integrin αV, or integrin β3 (Santa Cruz Biotechnology) or their corresponding scramble siRNAs. 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. Then, cells were incubated with 10% heat-inactivated FBS for 24 h, followed by 24 h in serum-free medium and then treated or not with CCN2(IV).

Animal model

Studies were performed in adult male C57BL/6 mice (9–12 weeks old, 20 g; Harlan Interfauna Ibérica) and maintained at local animal facilities. 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. C57BL/6 mice received a single intraperitoneal injection of recombinant CCN2(IV) (endotoxin levels <0.01 units; Preprotech) dissolved in saline at the dose of 2.5 ng/g of body weight as described (Sanchez-Lopez et al., 2009), and studied 24 h later ($n = 8–10$ mice per group). To block EGFR activation, animals were treated with erlotinib (40 mg/kg/day) or its vehicle (10% Ethanol) at 24 h before CCN2 injection ($n = 8–10$ mice per group). Mice were sacrificed under anaesthesia (Ketamine-HCl/Xylazine-HCl) and then kidneys were perfused *in situ* with cold saline before removal. We have previously demonstrated that CCN2(IV) administration did not cause tubular damage or fibrosis (Sanchez-Lopez et al., 2009).

Renal histology and immunohistochemistry

Immunohistochemistry was carried out on 3 μm paraffin-embedded kidney sections. Sections were deparafinized 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 (dilution) F4/80 (1:50), CD3 (1:300; Serotec), and p-EGFR$_{1173}$ (1:200; Cell Signalling) overnight at 4°C. 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.

Immunofluorescence was performed by incubating sections with 4% BSA/8% serum in PBS (for blockade), then anti-p-EGFR antibody (1:200; Dako), followed by AlexaFluor®633-conjugated antibody (1:200). The total number of positive stained cells was quantified in five randomly chosen fields (20×) using the Image-Pro Plus software. Data are expressed as positive stained area vs. total analysed area. Triplicate samples from each animal were examined in a blind manner.

ELISA for proinflammatory factors

CCL-2 and IL-6 protein levels were assayed by an ELISA kit (eBioscience), and quantified by comparison with a standard curve. Data are expressed as *n*-fold increase over the mean of control levels.

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 of total RNA primed with random hexamer primers. Multiplex real-time PCR was performed using Applied Biosystems expression assays mouse CCL-2 Mm00441242_m1 and mouse IL-6 Mm00446 190_m1. Data were normalized to 18S eukaryotic ribosomal RNA 4210893E (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.

Statistical analysis

All results are expressed as mean ± SEM. Differences between agonist-treated groups and controls were assessed by Mann–Whitney test. $P < 0.05$ was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0).

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