CCN2/CTGF increases expression of miR-302 microRNAs, which target the TGFβ type II receptor with implications for nephropathic cell phenotypes

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Summary

Signalling interplay between transforming growth factor-β (TGFβ) and CCN2 [also called connective tissue growth factor (CTGF)] plays a crucial role in the progression of diabetic nephropathy and has been implicated in cellular differentiation. To investigate the potential role of microRNAs (miRNAs) in the mediation of this signalling network, we performed miRNA screening in mesangial cells treated with recombinant human CCN2. Analysis revealed a cohort of 22 miRNAs differentially expressed by twofold or more, including members of the miR-302 family. Target analysis of miRNA to 3′-untranslated regions (3′-UTRs) identified TGFβ receptor II (TβRII) as a potential miR-302 target. In mesangial cells, decreased TβRII expression was confirmed in response to CCN2 together with increased expression of miR-302d. TβRII was confirmed as an miR-302 target, and inhibition of miR-302d was sufficient to attenuate the effect of CCN2 on TβRII. Data from the European Renal cDNA Biopsy Bank revealed decreased TβRII in diabetic patients, suggesting pathophysiological significance. In a mouse model of fibrosis (UUO), miR-302d was increased, with decreased TβRII expression and aberrant signalling, suggesting relevance in chronic fibrosis. miR-302d decreased TGFβ-induced epithelial mesenchymal transition (EMT) in renal HKC8 epithelial cells and attenuated TGFβ-induced mesangial production of fibronectin and thrombospondin. In summary, we demonstrate a new mode of regulation of TGFβ by CCN2, and conclude that the miR-302 family has a role in regulating growth factor signalling pathways, with implications for nephropathic cell fate transitions.

Key words: MicroRNA, Nephropathy, TGFβ, CCN2/CTGF

Introduction

Cell fate differentiation is critically important in development and increasingly implicated in numerous disease states. Excessive deposition of extracellular matrix is the common end point of many forms of kidney disease, with myofibroblast cells understood to be principal effectors of matrix accumulation. A predicted source of myofibroblasts is by derivation from resident renal fibroblasts and epithelial cells, in a process known as epithelial to mesenchymal transition (EMT), analogous to developmental cell fate transitions. Transforming growth factor-β (TGFβ) is known to be involved in the induction of matrix deposition and mesenchymal behaviour of epithelial and fibroblast cell types (Lan, 2003; Yang and Liu, 2001). Significant efforts are focused on the role that TGFβ and its mediators, including Connective Tissue Growth Factor (CTGF)/CCN2, play during the progression of diabetic nephropathy. In this context, TGFβ expression is increased (Sharma et al., 1997) and promotes fibrosis (Douthwaite et al., 1999; MacKay et al., 1989); however, it is evident that it also has a complex pleiotropic role. Increased expression of CCN2 is a confounding feature of the microenvironment that modulates responses in the mesangium during the initiation and progression of disease (Riser et al., 2000; Wahab et al., 2001). The nature of its structural organisation has led to the emerging view that CCN2 functions as a matricellular regulator (for reviews, see Brigstock, 2010; Holbourn et al., 2008; Shi-Wen et al., 2008). A better understanding of the consequences of TGFβ signalling in this CCN2-rich microenvironment would represent a significant advance in our understanding of the patho-mechanisms of nephropathy. A modulatory effect of CCN2 on TGFβ superfamily signalling has been defined in various contexts including embryonic development (Abreu et al., 2002; Shi-wen et al., 2006), skin fibrosis (Mori et al., 1999; Nakerakanti et al., 2011), diabetic nephropathy (Nguyen et al., 2008) and mesangial cell dysfunction (O'Donovan et al., 2012); while coordinate expression of TGFβ and CCN2 has been demonstrated in glomerulonephritis and diabetic nephropathy (Ito et al., 2010). Studies have also highlighted the cooperative nature of CCN2 and TGFβ in the promotion of fibrosis in animal models (Wang et al., 2011). In the kidney, several microRNAs (miRNAs) are preferentially expressed, with increasing appreciation of a role for miRNAs in nephropathy (Kato et al., 2007; Wang et al., 2008). In some contexts, modulation by miRNAs of CCN2 expression has been investigated, but to date no data exists on CCN2 mediated miRNA expression in any disorder. We demonstrate a mode of regulation of TGFβ signalling by CCN2 through increased expression of the miR-302 cluster. We show that members of this microRNA family induced by CCN2 can negatively regulate expression of TGFβ receptor II (TβRII), with resulting alteration in

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the balance of signalling pathways activated *in vitro*. We use a miR-302 mimic to abrogate *in vitro* development of EMT and present evidence for re-capitulation of key *in vitro* findings *in vivo*.

Results and Discussion

CCN2 induces microRNAs targeting the TGFβ type II receptor (TβRII)

We investigated miRNA expression in primary human mesangial cells (HMCs) treated with recombinant human CCN2 (rhCCN2) using a Taqman low density array. To confirm the biological activity of the rhCCN2 used, HMCs were first treated with the growth factor for a short timecourse and increased extracellular-signal-regulated kinase (ERK) and P38 phosphorylation was found, confirming functional activity (supplementary material Fig. S1). A cohort of miRNAs differentially expressed in response to CCN2 was determined (Fig. 1) and analysed. Pathway analysis of predicted miRNA targets identified signalling networks (supplementary material Table S1) including TGFβ signalling, MAP kinase signalling and extracellular matrix-receptor interactions. Increased expression of three members of the same miRNA family (miR-302b/c/d) was noted and target analysis suggested TβRII as a miR-302 target; TβRII expression was determined in HMCs treated with rhCCN2 and decreased TβRII mRNA was confirmed concomitant with increased miR-302d expression (Fig. 2A,B). Changes in miR-302d expression in response to rhCCN2 exhibited a dose and time dependent response, persistent to 48 hours (supplementary material Fig. S2) and changes in TβRII in response to rhCCN2 persisted to 96 hours (Fig. 2C). Transfection of full length CCN2 also increased miR-302d and decreased TβRII (Fig. 2D,E). Alignment of the 3′UTR of the TGFBR2 gene with miR-302s illustrated potential seed regions for microRNA binding (not shown). To determine if TGFBR2 was a true miR-302 target, we co-transfected a TGFBR2 3′UTR luciferase construct into HEK-293T cells with silencing (Anti-miR) or native miR-302d oligos (Pro-miR) (Fig. 2F). The effect of rhCCN2 on luciferase activity was also determined. Consistent with TGFBR2 being a miR-302d target, inhibition of the microRNA or introduction of a mimic altered luciferase activity, but the effect was modest. The experiment was repeated in more readily transfectable HeLa cells with similarly robust alterations in luciferase activity observed (supplementary material Fig. S3). rhCCN2 was sufficient to decrease luciferase activity in both cell lines, demonstrating regulation of TβRII expression via miR-302. In HMCs, Pro-miR or Anti-miR was sufficient to alter miR-302d levels (Fig. 2G) and consequently moderate the effect of rhCCN2 on TβRII (Fig. 2H,I).

Modulation of miR-302 results in altered signalling responses

Increased TGFβ expression has been linked to increased extracellular matrix production and fibrosis in diabetic nephropathy (Wu and Derynck, 2009; Ziyadeh, 2004). TGFβ signalling via specific serine/threonine kinase receptors and Smad effectors is well established, but it is also accepted that the proteomic composition of a cell influences the response to TGFβ signalling (Massagué and Chen, 2000). 'Context-dependent' events control not only canonical Smad2/3-dependent pathway responses but also the activation of Smad2/3-independent pathways regulating additional TGFβ responses (Derynck and Zhang, 2003). We next investigated CCN2/miR-302d as a context dependent regulator of signalling responses. Canonical TGFβ signalling requires binding of the ligand to TβRII, dimerisation of TβRII and TβRI, cross phosphorylation, and Smad2/3 recruitment (Massagué and Chen, 2000). Our observation of decreased TβRII expression suggested pathways independent of TβRII. We have previously described an shift in TGFβ signalling in the presence of CCN2, where canonical Smad based signalling is repressed by CCN2 and there is a resulting increase in non-canonical MAP kinase based signalling (O'Donovan et al., 2012). To evaluate the importance of CCN2 driven expression of miR-302s in this context, we manipulated miR-302d levels. Introduction of Pro-miR was sufficient to decrease TβRII levels and partially attenuate Smad3 activation (Fig. 2J, quantified in supplementary material Fig. S4), evidence for functional significance of miR-302 in regulation of TGFβ signalling. Conversely, transfection of Anti-miR was not associated with changes in canonical TGFβ signalling (Fig. 2K, quantified in supplementary material Fig. S4). We then transfected CCN2 and Pro-miR into HEK-293T cells expressing a Smad binding element (SBE) secreted alkaline phosphatase reporter (SEAP). Overexpression of CCN2 was sufficient to increase miR-302d levels, decrease TβRII and decrease TGFβ-induced SBE activity (Fig. 2L). Similarly transfection of Pro-miR resulted in decreased TβRII and SBE activity (Fig. 2M), demonstrating functional relevance of CCN2/miR-302 for SBE containing TGFβ target genes.

miR-302d is increased in UUO, concomitant with decreased TβRII and altered signalling responses

Data from the European Renal cDNA Biopsy Bank (Schmid et al., 2006) shows a decrease in TβRII in diabetic nephropathy patients versus controls (not shown), suggesting pathophysiological relevance of decreased TβRII expression. To evaluate a potential

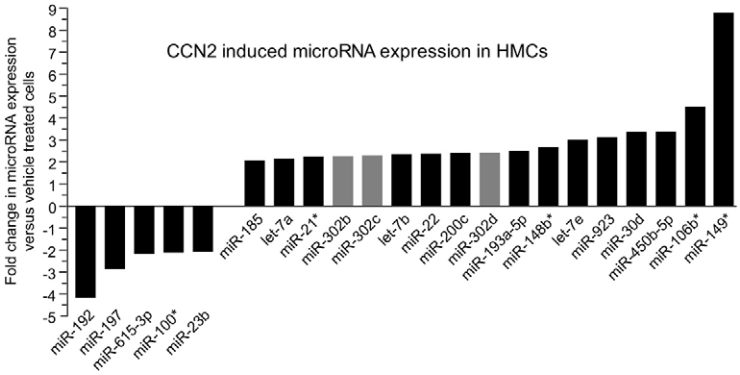


Fig. 1. CCN2 induces differential miRNA expression in HMCs. Primary HMCs were treated with rhCCN2 or vehicle for 24 hours and RNA containing the small RNA fraction was isolated. A TaqMan low-density array determined 22 miRNAs that were differentially expressed by twofold or more in response to rhCCN2. Three members of the miR-302 family showed an increase in expression (grey bars). Data represent the change in miRNA expression in a pooled sample from three biological replicates per condition. MicroRNAs with an asterisk are 'star' variants from the non-dominant arm of the miRNA duplex, which are often not integrated into the RNA induced silencing complex.

role for miR-302/TβRII in renal fibrosis we examined a 10 day unilateral ureteral obstruction (UUO) model. UUO induces pathogenic markers of kidney fibrosis including interstitial myofibroblast accumulation, matrix accumulation and tubular atrophy. Seed regions between mouse miR-302 family members and the orthologous mouse TGFBR2 gene were found by alignment (not shown). We measured miR-302d in renal tissue from sham operated and ligated mice, and contralateral kidneys.

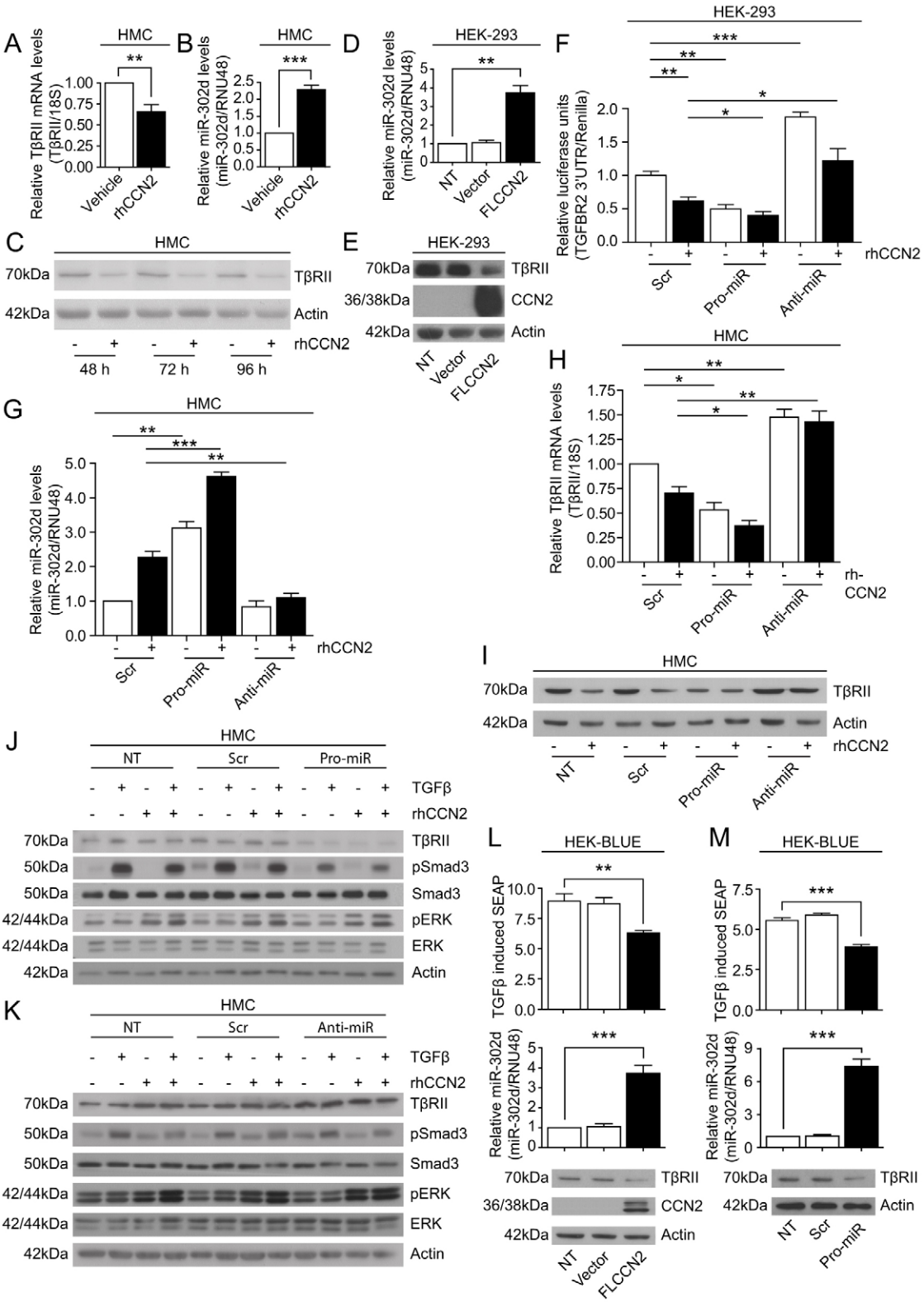


Fig. 2. See next page for legend.

Increased deposition of collagens and interstitial matrix protein was confirmed histologically in ligated kidneys by Picosirius Red and Masson's Trichrome (Fig. 3A, quantified in supplementary material Fig. S5A,B) in addition to increased fibronectin (FN-1) and thrombospondin 1 (TSP1), markers of fibrotic damage (Fig. 3B, quantified in supplementary material Fig. S5C,D). TGFβ mRNA and protein levels were found to be increased in ligated kidneys samples, indicating normal fibrotic pathophysiology (Fig. 3C), while miR-302d levels were also found to be increased in ligated kidney versus sham controls (Fig. 3D). Following on this observation, we examined expression of TβRII and major signalling components (Fig. 3E–G, quantified in supplementary material Fig. S5E–I) and carried out additional quantification of tubulin levels to confirm equal loading of protein (supplementary material Fig. S6). Consistent with increased expression of miR-302d, increased CCN2 and decreased TβRII was observed in ligated kidneys versus both non-ligated and sham operated controls. Canonical TGFβ effectors Smads 2 and 3 illustrated an apparent dichotomy; while both Smad2 and Smad3 were increased in ligated kidneys, only Smad3 was phosphorylated. Smad2 phosphorylation was decreased in ligated versus control, indicating that while increases in Smad2 are present, activation is attenuated. Noting the decreased expression of TβRII in UUO, we proceeded to delineate a role for receptor II independent and non-canonical signalling in the induction of fibrosis. Cells were with a kinase dead TβRII construct (TβRII K227R) and examined expression of fibronectin, thrombospondin and signalling mediators in response to TGFβ. While transfection of K227R was sufficient to abolish Smad phosphorylation, increased expression of fibronectin and thrombospondin still occurred, in addition to an apparent compensatory increase in ERK activation (Fig. 3H, left) To determine the functional importance of CCN2/ERK regulation of fibronectin and thrombospondin in this Smad-depleted system, we pre-treated TβRII K227R transfected cells with PD-98059 (a MEK inhibitor) or FG-3019 (a polyclonal antibody directed against CCN2) prior to TGFβ stimulation (Fig. 3H, right). PD-98059 was sufficient to partially attenuate TGFβ induced fibronectin and thrombospondin, this effect was more marked upon pre-transfection with K227R. Similarly, FG-3019 was sufficient to attenuate fibronectin induction in K227R transfected cells.

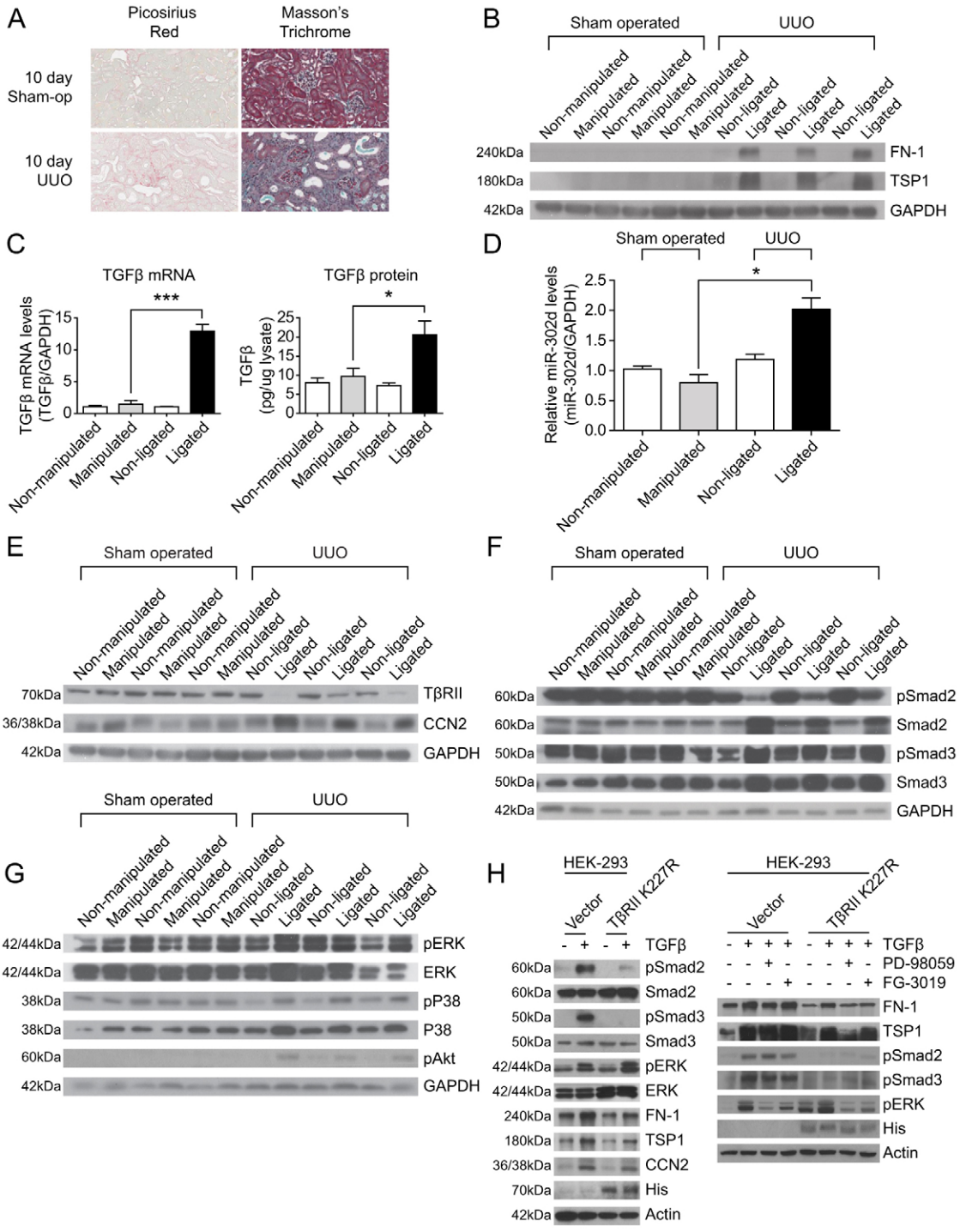
Fig. 2. TβRII is validated as an miR-302 target and is decreased by CCN2-induced miR-302, with implications for signalling and transcriptional responses. (**A**,**B**) Treatment of HMCs with rhCCN2 (25 ng/ml) results in decreased TβRII mRNA expression concomitant with increased expression of miR-302d. (**C**) Treatment of HMCs with rhCCN2 resulted in sustained decreased expression of TβRII to 96 hours. (**D**,**E**) The effect of endogenously produced CCN2 was confirmed when transfection of FLCCN2 into HEK-293 cells increased miR-302d levels and decreased TβRII. (**F**) HEK-293 cells were transfected with *TGFBR2* 3′-UTR and *Renilla* luciferase-containing construct for 24 hours and treated with rhCCN2. Co-transfection with pro-miR-302d (Pro-miR) decreased 3′-UTR luciferase activity, whereas transfection with anti-miR-302d (Anti-miR) increased luciferase activity, confirming *TGFBR2*/TβRII as an miR-302 target. Treatment of transfected cells with rhCCN2 was simultaneously sufficient to decrease luciferase activity, demonstrating that CCN2 can negatively regulate TβRII through miR-302. Data represent means±s.e.m. of $n=3$ experiments. (**G**,**H**) Transfection of HMCs with Pro-miR and Anti-miR was sufficient to alter miR-302d levels, with inverse changes in TβRII mRNA levels. The effect of rhCCN2 on miR-302d and TβRII mRNA was attenuated by Anti-miR, confirming the regulatory mechanism of CCN2. (**I**) Anti-miR was sufficient to reverse the effects of rhCCN2 on TβRII protein, whereas Pro-miR further dampened TβRII production in HMCs treated with rhCCN2. (**J**) TGFβ (10 ng/ml) and CCN2 co-treatment of HMCs results in dampened Smad3 activation, whereas non-canonical ERK signalling is increased. Transfection of cells with Pro-miR ablated the ability of TGFβ to activate canonical signalling but did not alter other signalling responses. (**K**) Conversely, transfection of HMCs with Anti-miR did not alter Smad or ERK activation. (**L**,**M**) Transfection of FLCCN2 or Pro-miR results in negative Smad2/3 transcriptional activity by SBE-dependent secreted alkaline phosphatase activity, where FLCCN2 increased miR-302d and decreased TβRII in HEK-Blue reporter cells and Pro-miR similarly decreased transcriptional response. All real-time qRT–PCR data represent means±s.e.m. of $n=3$ experiments with 3 replicates per experiment. All blots are representative of $n=3$ experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Abbreviations: NT, non-transfected control; Scr, scrambled oligo control; SEAP, secreted alkaline phosphatase.

miR-302d can regulate TGFβ induced EMT and blunt mesenchymal phenotype via TβRII inhibition

Members of the miR-302 family have been primarily associated with embryonic cell differentiation (Lin et al., 2010), and a recent study has demonstrated a role for miR-302 family members in the re-programming of human fibroblasts to pluripotent stem cells (Subramanyam et al., 2011). Differentiation of embryonic stem cells requires discreet changes including loss of E-cadherin, increased vimentin, *de novo* smooth muscle actin synthesis and increased gelatinase activity (Eastham et al., 2007; Rubio et al., 2008). Evidence of cellular plasticity, characterised as epithelial to mesenchymal transition (EMT) has been described in *in vitro* models of renal fibrosis (Iwano et al., 2002; Okada et al., 2001) and *in vivo* (Rastaldi et al., 2002). We examined the effect of miR-302 on renal cells in this context by transfecting miR-302d into tubular epithelial and glomerular mesangial cells, and determined expression of classical markers of plasticity and fibrosis in response to TGFβ. In HMCs TGFβ induced fibronectin and thrombospondin was attenuated by pre-transfection with Pro-miR (Fig. 4A, quantified in supplementary material Fig. S7A). To determine whether this effect was modulated by negative regulation of TβRII, we transfected cells with a vector encoding wild-type TβRII (WT TβRII) with or without Pro-miR, and treated with TGFβ. Pro-miR transfection was sufficient to sustain increased miR-302d levels in HEK-293 cells (Fig. 4B) and the attenuation of fibronectin and thrombospondin by miR-302d was reversed by WT TβRII (Fig. 4C, quantified in supplementary material Fig. S7B). In HKC8 tubular epithelial cells transfection of CCN2 resulted in increased miR-302d levels (Fig. 4D) and decreased TβRII expression (Fig. 4E), demonstrating consistent CCN2 regulation of miR-302/TβRII between mesangial and epithelial cells. Transfection of Pro-miR resulted in sustained miR-302d levels (Fig. 4F) and we investigated the effect of miR-302 on TGFβ induced EMT. Pro-miR attenuated TGFβ induced vimentin and N-cadherin, while restoring levels of E-cadherin and ZO-1 (Fig. 2G, quantified in supplementary material Fig. S7C). Transfection of WT TβRII was sufficient to reverse this effect. Transfection of Pro-miR also abrogated TGFβ induced vimentin reorganisation and loss of junctional ZO-1 expression in HKC8 cells (supplementary material Fig. S8). Relevance for CCN2 induced miR-302 as a negative regulator of EMT was confirmed where transfection of native CCN2 into epithelial cells was sufficient to attenuate TGFβ induced vimentin and reduced E-cadherin (supplementary material Fig. S9).

Regulation of adult cell signalling by miR-302

To date the miR-302 family have been reported to have roles in the regulation of embryonic cell differentiation and as potential tumour biomarkers (Lin et al., 2010; Murray et al., 2011). MiR-302 miRNAs share homology with miR-430 family members, which have known TGFβ signalling targets (Rosa et al., 2009). Here we report that in adult mesenchymal cells, members of the miR-302 family induced by CCN2 can negatively regulate expression of the type II TGFβ receptor with implicit implications for the activation of canonical signalling and downstream transcription by TGFβ, findings in line with those reported by others – Subramanyam and colleagues found that promotion of miR-302 was sufficient to dampen TβRII and Smad2/3 activation (Subramanyam et al., 2011) while Lipchina and colleagues demonstrated that miR-302/367 decreased BMP-Id1 transcription and Lefty1/2 expression – distant members of



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the TGFβ superfamily (Lipchina et al., 2011). Similarly, regulation by miR-302/427/430 families of Lefty1/2 in mesodermal fate specification has been described (Rosa et al., 2009); the data presented here shows that regulation of TGFβ signalling by members of the miR-302 family is not confined to embryonic stem cell behaviour/differentiation, but also regulates behaviour in adult cells with pathogenic significance.

Decreased TβRII and divergent signalling in the UUO mouse

We report that in a chronic model of tubulointerstitial fibrosis, expression of CCN2 and miR-302 is increased while TβRII expression is decreased in parallel with divergent Smad activation. This interesting finding lends support to a hypothesis of differential roles for Smad2 and Smad3 in the regulation of profibrotic responses. Specific gene responses have been found to be dependent on either Smad2 or Smad3 (Phanish et al., 2006), and multiple studies have demonstrated that deletion of Smad3 attenuates fibrosis (Roberts et al., 2006; Sato et al., 2003) while the same does not apply for Smad2 (Yang et al., 2010). Further, a recent study by Meng and colleagues demonstrated a protective role for Smad2 in UUO, deletion being associated with greater fibrosis by enhanced Smad3 phosphorylation and auto-induction of TGFβ, while overexpression attenuated Smad3 phosphorylation and collagen I (Meng et al., 2010). More recently again, a study by the same authors evaluated disruption of TβRII in UUO. This resulted in incomplete attenuation of collagen I, α-smooth muscle actin and fibronectin production, but was associated with an enhanced inflammatory response (Meng et al., 2012). Increased expression of TGFβ receptor I and II mRNA has been reported in UUO (Liu et al., 2011; Sutaria et al., 1998) but these findings do not preclude receptor turnover or degradation by microRNAs including miR-302s. As described above (Fig. 3C), we measured increased expression of TGFβ in UUO; however, a number of studies have shown that TGFβ negatively regulates the expression of TβRII (Meng et al., 2011; Truty et al., 2009) so it seems possible that a similar mechanism is found in the UUO mouse. We propose that this represents a negative regulatory loop in TGFβ signalling. It is clear that many different mechanisms independent of TGFβ exist by which ERK, p38 and Akt can be regulated in fibrosis; these likely include a role for TGFβ/CCN2/miR-302 negative feedback impeding canonical signalling. It has previously been reported that CCN2 can regulate pathological features of fibrosis via ERK (Chen et al., 2004; Ding et al., 2011; Fuchshofer et al., 2011) and recently in UUO, inhibition of CCN2 with a FG-3019 has lessened collagen deposition and fibrosis severity (Wang et al., 2011). Specific inhibition of CCN2 and MEK/ERK demonstrated that a switch in signalling mediator occurs from Smad dependent to Smad independent in the presence of the kinase dead TβRII. Paralleling this switch we observed continued increased expression of fibronectin and thrombospondin; in the absence of Smad signalling fibrotic responses persist, supporting the findings of Pannu and colleagues in dermal fibroblasts (Pannu et al., 2007). These findings strengthen the hypothesis that CCN2/ERK can regulate the fibrotic response. Intriguingly a recent study has demonstrated exacerbated aortic growth in the presence of ablated Smad signalling in a model of Marfan's syndrome, defining a critical role for non-canonical TGFβ signalling in a disease context (Holm et al., 2011).

Fig. 3. Increased expression of miR-302d is found in 10-day-ligated UUO mouse kidney tissue with prevalence of canonical and non-canonical TGFβ signalling; *in vitro* use of kinase-dead TβRII demonstrates TβRII-independent induction of fibrotic markers and impetus upon CCN2/ERK induction. (**A**) Increased deposition of collagen was confirmed by Picrosirius Red staining of kidney sections and increased connective tissue deposition was evidence by Masson's Trichrome staining, confirming induction of kidney fibrosis. (**B**) Increased induction of fibronectin (FN-1) and thrombospondin 1 (TSP1) production was confirmed in the ligated kidney. (**C**) Real-time PCR analysis revealed increased TGFβ mRNA in mouse RNA samples from ligated kidneys (left; data represent means±s.e.m. from $n=4$ animals), whereas TGFβ protein was assessed by ELISA and found to be increased in the ligated kidneys (right; data represent means±s.e.m. for $n=4$ animals). (**D**) Real-time PCR analysis found increased miR-302d levels in the ligated kidneys, as a proxy readout for miR-302 activity. Data represent means±s.e.m. for $n=4$ animals. (**E**) The expression of TβRII and CCN2 was determined and found to be decreased and increased (respectively) in the ligated kidneys, suggesting potential recapitulation of *in vitro* observations from HMCs. (**F**) Evaluation of canonical signalling of Smad2/3 found divergent phosphorylation of Smad2 and Smad3, with only Smad3 phosphorylation being increased. (**G**) Increased non-canonical signalling induction in the form of increased ERK, p38 and Akt phosphorylation was also observed. (**H**) To further examine the importance of TβRII in the induction of fibrosis a kinase-dead dominant-negative TβRII construct (TβRII K227R) was transfected into HEK-293 cells (left panel). Induction of fibronectin and thrombospondin by TGFβ still occurred in the presence of the TβRII K227R; simultaneously, a compensatory increase in ERK activation and production was observed. To dissect the importance of CCN2/ERK in this effect, TβRII K227R-transfected cells were pre-treated with either the MEK inhibitor PD-98059 (10 μM) or a monoclonal antibody to CCN2 FG-3019 (10 μg/ml) prior to TGFβ treatment (right panel). Both the MEK inhibitor and the antibody to CCN2 were sufficient to further attenuate fibronectin production in the presence of TβRII K227R, demonstrating a role for CCN2/ERK in regulation of fibrotic markers in the absence of TβRII. All blots for kidney tissue expression are from $n=3$ animals from both groups; all other blots are representative of $n=3$ experiments *in vitro*. $^{*}P<0.05$, $^{***}P<0.001$. op, operated.

miR-302/CCN2 as regulators of EMT

Subramanyam and colleagues have recently demonstrated in HaCat cells that miR-302b inhibited TGFβ induced EMT by way of restoring E-cadherin and ZO-1 expression (Subramanyam et al., 2011). A further recent study by Lipchina and colleagues demonstrated in addition to targeting TGFβ signalling, miR-302 promoted BMP signalling by targeting BMP inhibitors TOB2, DAZAP2 and SLAIN1 in human embryonic stem cells, with the effect of maintaining pluripotency (Lipchina et al., 2011). In this study, we found that miR-302 could attenuate induction of selective markers of fibrosis by TGFβ, an effect which was dependent on inhibition of TβRII. Taken together, it is apparent that miR-302 can regulate developmental EMT and the data shown here suggests similar function in disease associated EMT. Notwithstanding current controversies surrounding the role of EMT in renal fibrosis, a consensus is slowly emerging that concedes a partial differentiation of epithelial cells in tubulointerstitial fibrosis that may be characterised by intermediate phenotypical changes (Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2010), although it remains to be proven if 'partial-EMT' is capitulated in renal fibrosis (Kriz et al., 2011; Zeisberg and Duffield, 2010). Pathologically, the most salient feature of nephropathy is glomerular and tubular hypertrophy. The complexities of TGFβ signalling in this microenvironment are slowly emerging – in this context it is possible that CCN2 induced miR-302 regulates epithelial cell differentiation and hypertrophy. Burns and colleagues have previously reported that CCN2 induced a dose dependent change in cell morphology, with significantly

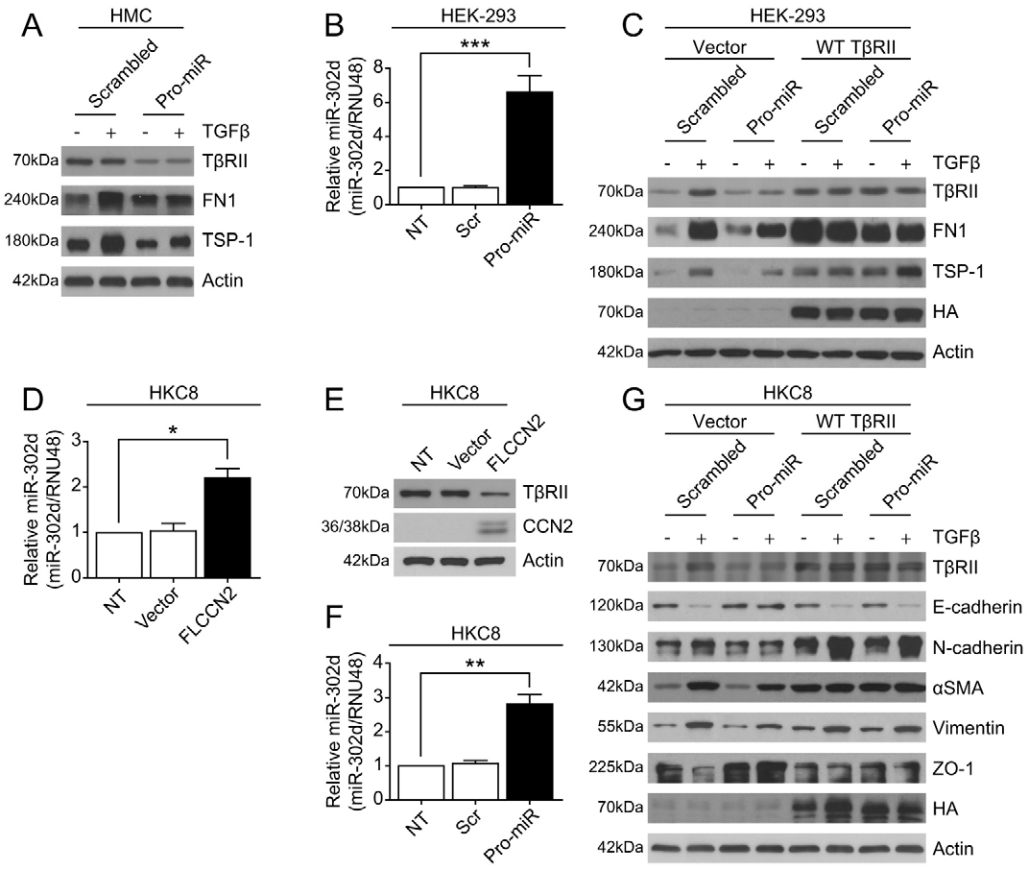

Fig. 4. miR-302d reverses TGFβ-induced expression of markers of fibrosis in HMCs and inhibits EMT in renal epithelial cells, an effect that can be reversed by overexpression of TβRII. (**A**) In HMCs, pre-transfection of cells with pro-miR-302d (Pro-miR) attenuated TGFβ (10 ng/ml)-induced expression of fibronectin (FN-1) and thrombospondin (TSP-1). (**B**) Transfection of Pro-miR into HEK-293 cells results in sustained miR-302d levels. (**C**) Pro-miR was then co-transfected into HEK-293 cells with a wild-type TβRII construct (WT TβRII). WT TβRII was sufficient to increase FN1 and TSP-1 basal levels but did not significantly enhance their response to TGFβ compared with vector co-transfected control. The role of CCN2/miR-302 on TGFβ-induced EMT in renal cells was then examined. (**D**) Transfection of FLCCN2 into HKC8 cells was sufficient to increase miR-302d levels; (**F**) similarly, transfection of Pro-miR resulted in sustained miR-302d levels and (**E**) transfection of cells with FLCCN2 decreased TβRII levels. (**G**) Pro-miR and WT TβRII were transfected into epithelial cells and the EMT responses to TGFβ examined. Pro-miR restored E-cadherin and ZO-1 levels reduced by TGFβ, partially attenuated α-smooth muscle actin (αSMA) and vimentin induction and attenuated N-cadherin production. The effect of miR-302 on αSMA, ZO-1 and N-cadherin was substantially reversed by transfecting in WT TβRII. All real-time qRT-PCR data represent means±s.e.m. of $n=3$ experiments with three replicates per experiment. All blots are representative of $n=3$ experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Abbreviations: NT, non-transfected control, Scr, scrambled oligo control.

different characteristics to that of TGFβ-induced EMT (Burns et al., 2006). The authors found that instead of reducing E-cadherin expression, CCN2 induced a peri-nuclear redistribution of the normally junctional protein. In this study, the divergent regulatory effect of CCN2 on E-cadherin/vimentin/α-smooth muscle actin supports a hypothesis where CCN2 functions as a 'partial-EMT' mediator.

Recent findings from Fragiadaki and colleagues demonstrated that CCN2 in itself is not sufficient to promote fibrosis (Fragiadaki et al., 2011). This observation, along with other studies has strengthened the view that CCN2 functions as a co-ordinator of other profibrotic factors including TGFβ. MicroRNAs have significant promise as novel therapeutics, for example antagomirs have been shown to successfully target microRNA in the kidneys (Krützfeldt et al., 2005). The observation that miR-302 is sufficient to attenuate expression of markers associated with fibrosis in a myofibroblast cell and stabilise epithelial phenotype suggests that manipulating the expression of miR-302 in the context of renal fibrosis may confer therapeutic benefit.

Materials and Methods

Animals and histology

Procedures were licensed by the Irish Department of Health and approved by the UCD Animal Research Ethics Committee. Male C57Bl/6J mice aged 10–12 weeks were placed in two groups: UUO and sham-operated. Animals in the UUO group were anaesthetised, received midline laparotomy; the left ureter was located and ligated. Animals in the sham group had the left ureter exposed and manipulated. On day 10, mice were harvested, UUO was confirmed by dilation of the renal pelvis, and the renal capsule was removed and tissue sections snap frozen. Paraffin embedded sections (5 μM) were stained with Picosirius Red and Masson's Trichrome and scored blindly from 1–5 with 1 representing collagen deposition or matrix distribution and 5 representing 5 fold or more collagen accumulation and >75% of the cortex having more matrix distribution than normal.

Tissue culture

Primary human mesangial cells (Lonza, Basel, Switzerland) were cultured in MCDB-131 media supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/1 mg/ml streptomycin and 2 mM L-glutamine (all from Invitrogen, Paisley, UK). HEK-293T/17, HKC8 proximal tubular epithelial cells and HeLa cells (all from American Type Culture Collection) were cultured in DMEM (Lonza) supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. HEK-Blue TGFβ cells (Invivogen, Toulouse, France) were cultured in DMEM supplemented with 4.5 g/L D-glucose, 10% FBS, penicillin/streptomycin, L-glutamine, 30 μg/ml Blasticidin, 200 μg/ml Hygromycin and 100 μg/ml Zeocin

(selective antibodies from Invivogen). Cultures were maintained at 37℃ in an environment of 5% CO_2/95% air, and were serum restricted (0.2% FBS) for 24 hours prior to all stimulations. Cells were treated with 10 ng/ml TGFβ1 (Promokine, Heidelberg, Germany), 25 ng/ml rhCCN2 (Fibrogen Inc., South San Francisco, CA) or both together, for 24 hours to determine miRNA expression changes, 48/72/96 hours to determine changes in TβRII, 24 hours for EMT/fibrotic marker expression and 30/180 minutes to determine changes in TGFβ signalling. MEK/ERK activity was inhibited by adding PD-98059 (Merck, Whitehouse Station, NJ) at 10 μM for 1 hour prior to treatment with TGFβ. TGFβ-induced CCN2 activity was inhibited by adding FG-3019 (Fibrogen Inc.) at 10 μg/ml for 1 hour prior to treatment with TGFβ.

RNA isolation, Taqman low density array and qRT-PCR

Total RNA with intact small RNA fraction was isolated using a miRVana kit (Ambion, Austin, TX). Total RNA from mouse renal tissue was isolated with an RNeasy Mini kit (Qiagen, Hilden, Germany). Single strand cDNA was reverse transcribed using primer pools directed against 850 microRNAs, qRT-PCR was carried out in a Taqman low density array on a 7900HT Real-time PCR system (all from Applied Biosystems, Foster City, CA). Data was normalised using endogenous small nuclear RNAs (RNU42/44/48) with a cycle threshold (Ct) of <33 applied. Expression of TβRII mRNA, mouse TGFβ mRNA and miR-302d was determined by Taqman qRT-PCR (assays Hs00234253_m1*, Mm01178820_m1 and 244178_mat, respectively, normalised to 18S rRNA or RNU48, Applied Biosystems).

miRNA mimic/inhibitor, pCMV5B-TGFβ receptor II wt/K227R and FLCCN2

Pro-miR-302d (synthetic, MSY0000718) and Anti-miR-302d (inhibitory, MIN0000718) oligonucleotides, and scrambled control oligonucleotides were from Qiagen; sub-confluent cells were transfected with 40 nM of Pro-miR, Anti-miR or scrambled control using Fugene HD (Promega, Madison, WI) diluted in Opti-MEM I for 24 hours prior to serum restriction and stimulation. TβRII -type (pCMV5B-TGFbeta receptor II wt, NO. 11766, Addgene, Cambridge, MA), TβRII K227R dominant negative (pCMV5B-TGFbeta receptor II K227R, NO. 11762, Addgene) and full length CCN2 (FLCCN2, Fibrogen Inc.) were transfected into cells using Fugene HD for 24 hours. Empty vectors were transfected into control cells for all vectors used.

Luciferase assays

HEK-293T/HeLa cells were co-transfected with a TGFBR2 3′UTR luciferase containing construct (SwitchGear Genomics, Menlo Park, CA) with either Pro-miR or Anti-miR and normalised with Renilla luciferase. Cells were then serum restricted prior to treatment with rhCCN2 (25 ng/ml) for 24 hours. Luciferase activity was quantified with a dual-luciferase reporter kit (Promega).

HEK-Blue TGFβ SEAP assay

HEK-Blue TGFβ cells were transfected with FLCCN2 or Pro-miR for 24 hours (or empty vector/scrambled RNA controls) using Fugene HD. Cells were then serum restricted prior to treatment with TGFβ for 24 hours. SBE dependent SEAP activity was quantified with Quanti-Blue substrate (Invivogen) by absorbance at 620 nM.

Protein extraction, electrophoresis and western blotting

Total protein was isolated from cells and kidney sections in modified radio immunoprecipitation (RIPA) buffer (Tris-HCl, NaCl, EDTA, Na-deoxycholate, sodium dodecyl sulphate, supplemented with protease/phosphatase inhibitor cocktails (Sigma)). Normalised protein samples were run in 8–12% polyacrylamide SDS gels, transferred to polyvinylidene fluoride (Millipore, Watford, UK), and probed for TβRII (Abcam, Cambridge, UK), phospho-Akt/Akt, phospho-p38/p38, phospho-Smad2/Smad2, phospho-Smad3/Smad3, phospho-ERK/ERK, phospho-Smad1/5/8 (all from Cell Signalling Technologies, Beverly, MA), CCN2 (Santa Cruz Biotechnology, Santa Cruz, CA), fibronectin, thrombospondin, N-cadherin (all from BD Transduction, Lexington, KY), E-cadherin (Abcam), ZO-1 (Zymed, San Francisco, CA), smooth muscle actin, vimentin and β-actin (all from Sigma).

Immunofluorescence

Confluent HKC8 cells were transfected with Pro-miR or scrambled control for 24 hours and serum restricted before being treated with TGFβ for 24 hours. Cells were fixed with 3.7% paraformaldehyde (EMS, Fort Washington, PA), permeabilised with 0.1% Triton X-100 (Sigma) and blocked with 5% Goat Serum (Sigma). Cells were then stained for ZO-1 and Vimentin (Texas Red anti-rabbit/mouse IgG secondary, Invitrogen) and counterstained with Hoechst 33342 (Invitrogen). Images were acquired with an Axiovert 200M or Imager.M1 microscope and processed with Axiovision 4.0 (Carl Zeiss, Jena, Germany).

Mouse TGFβ ELISA

An ELISA for TGFβ in lysates from 10 day sham operated and UUO ligated mice was carried out with a DuoSet ELISA kit (R&D Systems) to the manufacturer's instructions. A set of standards was created by diluting mouse TGFβ1 in serial twofold dilutions from 2000 pg/ml to 31.25 pg/ml. The absorbance values for the standards were used to generate a curve (R^2=0.9918) and a linear regression was performed and used to determine the concentration of TGFβ in the individual samples.

Bioinformatic analysis

miRNA targets were determined using TargetScan 5.0 [http://www.targetscan.org (Lewis et al., 2003)] and miRNA target gene alignments were retrieved from microRNA.org [http://www.microRNA.org (Betel et al., 2008)]. Common physiological target pathways for miRNAs were determined with DIANA-mirPath [http://diana.cslab.ece.ntua.gr/pathways/ (Papadopoulos et al., 2009)].

Statistical analysis

Graphs are expressed as mean +/− standard error. Densitometry was carried out with ImageJ software from the NIH. Statistical analysis of differences between groups was by one way ANOVA with post-hoc Tukey's test or Student's *t*-test, as appropriate. A P-value less than 0.05 was considered significant, analysis was carried out with GraphPad Prism software (GraphPad, San Diego, CA).

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