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Original Research

CCN3, a key matricellular protein, distinctly inhibits TGFβ1-mediated Smad1/5/8 signalling in human podocyte culture

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Abstract: Growth factors like TGFβ and CTGF (CCN2) plays a vital role in various cellular functions. TGFβ and CTGF are overexpressed in renal fibrosis. CTGF act as profibrotic stimuli to TGFβ. CCN3 is a member of CCN family which also comprises CCN1 (CYR61), CCN2 (CTGF), CCN4 (WISP-1), CCN5 (WISP-2) and CCN6 (WISP-3). CCN3 has been shown to antagonise CTGF. In this study, we investigated the role of CCN3 in TGFβ1-mediated signalling in human podocytes culture. This study describes the novel function of CCN3 in regulation of TGFβ1 mediated non-canonical Smad signalling in human podocytes culture. Experiments were conducted on conditionally immortalised human podocytes incubated with TGFβ1 (1.25ng/ml and 2.5ng/ml) and CCN3 (360ng/ml). Western blot study was performed to study signalling proteins. RT-PCR was performed to study alternative splicing of Fibronectin (Fn). Real time PCR was performed to look for gene expression of Fn and collagen IV and collagen I. TGFβ1 induced the Smad1/5/8, Smad3 and p38 phosphorylation and CCN3 downregulated the TGFβ1 induced Smad1/5/8 phosphorylation and did not affect Smad3 and p38 phosphorylation. In addition to this CCN3 induced alternative splicing of Extra domain A Fibronectin (EDA+Fn). CCN3 also induced collagen IV, Collagen I and Fn gene expression. This is the first evidence of downregulation of TGFβ-mediated activation of a Smad1/5/8 signalling pathway by CCN3 in human podocytes and in any cell type. Targeting CCN3-mediated events could provide exciting outcomes in the understanding of molecular mechanism of fibrosis.

Key words: Podocyte; TGFβ1; CCN3; CTGF; Fibrosis.

Introduction

Growth factors like TGFβ and CCN2 widely known as connective tissue growth factor (CTGF) play a vital role in various cellular functions. TGFβ and CTGF are overexpressed in renal fibrosis. CTGF act as profibrotic stimuli to TGFβ. CTGF has been extensively studied describing its role in fibrosis. Recently CCN3 (NOV-nephroblastoma overexpressed) has been shown to antagonise the activity of CTGF (1).

CCN is a family of six cysteine rich matricellular proteins which are named as CCN1 to CCN6 and has found to be involved in various important cellular processes such as cell proliferation, tumorigenesis, wound healing etc. CCN family comprises CCN1 (CYR61), CCN2 (CTGF), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3). Members of the CCN family are made up of an N-terminal secretory signal peptide followed by four conserved domains having homology to insulin-like growth factor binding protein, von Willebrand factor type C repeat, thrombospondin type 1 repeat, and a C-terminal domain (CT) with heparin-binding motifs and sequence similarity to the C termini of von Willebrand factor and mucin. CCN3 is another member of CCN family. CCN3 is also known as IGFBP- rP3 (Insulin-like growth factor binding protein- related protein 3) (2,3).

Podocytes are spectacular cell type which plays a critical role in filtration. In previous studies, it has been demonstrated that TGFβ1 significantly induces the Fn gene expression in conditionally immortalised human podocytes culture (4). In conditionally immortalised mouse podocytes TGFβ1 induces the Fn gene expression and protein expression (5).

TGFβ1 is a multifunctional cytokine that mediates diverse cellular functions in a cell type and context dependent manner. TGFβ1 imparts its effects by activating a distinct range of signalling pathway (15). TGFβ1 mediates its signalling by binding to its receptors which in turn recruits different proteins. The members of the TGFβ superfamily proteins binds to two distinct receptors types known as type II and type I receptors, both are required for signal transduction. There are five type II receptors and seven type I receptors (ALK1 to -7) present in mammals. The well-studied TGFβ1 signalling pathway is the Smad protein signalling pathway (7). TGFβ1 binds to its serine-threonine kinase receptor type II. Two molecules of each type II and type I receptors form a heterotetrameric complex. In this complex, the type II receptor then trans-phosphorylates the type I receptors. The type I receptor then recruits and phosphorylates different transcriptional Smad proteins.

Smad proteins are categorised in three subclasses which are receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads). Regulatory Smads are further divided into two subclasses Smad2 and Smad3 which are activated by TGFβ1 type I receptors (ALK5) and Smad1/5/8 acti-

vated by bone morphogenetic receptors. Smad1 is also activated by TGFβ1 type I receptor (ALK1) in endothelial cells (6,8,9). After activation, these R-Smads then forms complex with Smad4 (Co-Smad) and this complex then translocates to the nucleus and regulates the transcription of a target gene by binding to promoter region (9,10).

The Smad2 and Smad3 pathway is called as TGFβ mediated canonical signalling pathway whereas Smad1/5/8 is called as TGFβ mediated non-canonical Smad signalling pathway (11). TGFβ1 has been shown to mediate a vast range of cellular functions such as growth, proliferation, apoptosis, ECM production, migration and adhesion (12). The regulation of all these functions is critical for the healthy development and functioning of cells. TGFβ1 mediates these functions by regulating a diverse range of signalling pathways. Disrupted regulation of these signalling pathways leads to various pathologies.

In these studies, I have investigated the effect of CCN3 on TGFβ1 mediated signalling pathways, in the regulation of alternative splicing of EDA+Fn and other ECM proteins gene expression in human podocytes culture.

Materials and Methods

Cell culture

Conditionally immortalized human podocyte cell culture retrovirally transfected by temperature sensitive SV40 large T-antigen (Developed by Dr Moin Saleem, a kind gift from Jochen Raiser) were cultured as monolayer at the permissive temperature of 33°C in a humidified atmosphere of 5% CO_2 and 95% air, with RPMI 1640 medium supplemented with heat-inactivated 10% Fetal Calf Serum, L-glutamine (2mM)-penicillin (100U/ml)-streptomycin (100µg/ml) antibiotics, 5mM D-Glucose and insulin (5µg/ml)-transferrin (5µg/ml)-sodium selenite (5ng/ml). The medium was changed every alternate day. Confluent cells were passaged by aspirating the media and incubating with trypsin–EDTA solution (trypsin (5 g/l), Na2-EDTA (2 g/l), NaCl (8.5 g/l) for 4 minutes at 33°C temperature. Trypsin was neutralised with normal culture medium and cells were centrifuged at 350 x g for 6min at room temperature. The cell pellet was re-suspended in fresh culture medium. Viable cells were counted with 0.4% trypan blue dye exclusion method and were seeded at a density of 10,000 cells/cm^2. For experimental studies, podocytes were grown for 4 days at 33°C (permissive condition) and then at 37°C for 14 days (Non-permissive condition). All the experiments were conducted on overnight serum starved 80-90% confluent culture of terminally differentiated podocytes passages between 3 and 25.

Stimuli and inhibitors

Serum-starved differentiated podocytes at 37°C were treated with TGFβ1 2.5ng/ml (Sigma-Aldrich, Poole, Dorset, UK) or vehicle (0.1% w/v BSA) and for inhibitory studies serum-starved differentiated podocyte cells were pre and co-treated with CCN3 360ng/ml (Pepro Tech, NJ, USA) with or without TGFβ1 (2.5ng/ml or 1.25ng/ml) compared with vehicle (0.1% w/v BSA) for indicated period of time.

Western Blot Analysis

After the treatment, cells were washed once with ice-cold 1 x PBS and lysed by cell scraper in 70µl ice-cold lysis buffer Tris/HCl (20mM), NaCl (150mM), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM EDTA, phosphatase inhibitor cocktail (1x) and protease inhibitor cocktail (2x). Cell lysates were kept on ice for 15min and centrifuged at 10000 x g at 4°C for 10 min to remove cell debris. Supernatants containing protein were collected and the pellet containing cell debris was discarded. Supernatants containing proteins were either stored at -80°C for future use or was subjected to protein quantification. Total cellular protein concentration was determined using the colorimetric bicinchoninic acid protein assay (BCA) kit following manufacturer protocol.

The protein samples were prepared for western blotting under denaturing and reducing condition by heating at 70°C for 10min in a solution containing NuPAGE LDS Sample buffer (1x) and NuPAGE Sample Reducing Agent (1x). Equal amounts of cellular proteins were subjected to SDS-PAGE. Proteins were transferred onto PVDF membrane for 4h in NuPAGE transfer buffer at 30 V using the XCell II Blot Module (Life Technology). After transfer the blots were washed with TBS-T buffer [Tris-buffered saline/20mM Tris/HCl, 150mM NaCl and 0.1% (v/v) Tween 20) 5% (w/v)] on 3D gyratory rocker for 15 min. Blots were then blocked with TBS-T fat-free milk 5% (w/v) for 60min. Blots were washed for 10min x 3times with TBS-T. Blots were incubated with appropriate primary antibodies (Table 1) either in TBS-T with 5% (w/v) BSA (rabbit polyclonal antibodies) or in TBS-T with 5% (w/v) fat-free milk (Mouse Monoclonal antibodies) at 4°C overnight. Blots were washed for 10min x 3times with TBS-T buffer on a 3D gyratory rocker. Blots were incubated with horseradish peroxidase HRP-labelled secondary antibody for 1h at

Table 1. Antibody dilutions used for Western blotting.

Target protein	Size(KDa)	Antibody	Dilution	Cat. no	Supplier
EDA+ Fn	220	Mouse monoclonal	1:1000	ab 6328	Abcam
pSmad2	60	Rabbit monoclonal	1:1000	3108	Cell signaling
pSmad3	52	Rabbit monoclonal	1:1000	9520	Cell signaling
pSmad1/5/8	60	Rabbit polyclonal	1:1000	9511	Cell signaling
pP38	43	Rabbit polyclonal	1:1000	9211	Cell signaling
Antimouse IgG(Secondary Ab)	-	Rabbit	1:40	A9044	Sigma-Aldrich
Antirabbit IgG(Secondary Ab)	-	Rabbit	1:1000	7074	Cell signaling
α/β-Tubulin	55	Rabbit polyclonal	1:1000	2148	Cell signaling

room temperature at 3D gyratory rocker and developed with ECL Prime enhanced chemiluminescence western blotting detection system and visualised with Hyperfilm ECL photographic film developed by MI-5 X-ray film processor (VWR). Blots were stripped and re-probed for housekeeping tubulin protein as a loading control.

Western blot films were imaged by ImageQuant 300 Imager and ImageQuant Capture software (v1.0.0.4; GE Healthcare Life Sciences). Quantification of band density was done by Image count TL software (v1.0.0.4; GE Healthcare Life Sciences).

RNA extraction, reverse transcription and PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen UK Ltd) using manufacturer's protocol. All buffers for RNA extraction were provided as part of extraction kit. All disposable plastic ware were purchased as DNase and RNase free. Total cellular RNA concentration was then quantified by measuring the absorbance at 260nm and quality was measured by determining 260 to 230 ratio using NanoDrop Spectrophotometer. The extracted RNA was subjected to Reverse Transcription (RT) to synthesise cDNA. RT was performed to synthesise cDNA using Reverse Transcriptase system (Applied Biosystem, Foster City, CA, USA). The cDNA generated after the RT-PCR was subjected to conventional PCR amplification for EDA+/-Fn. The PCR reaction was performed using the Crimson Taq Polymerase (New England Biolabs) in a total volume of 25µl and the volume of cDNA template was 1µl from 1 in 10 diluted RT reactions. Each forward and the reverse primer was used at 20µM with 1.25 U polymerase per reaction. The inclusion of EDA exon was detected using a pair of primer binding constitutively spliced exon upstream and downstream of 270bp EDA exon. The PCR leads to inclusion of EDA exon which yields two products a 104bp band fragment corresponding to EDA-Fn and a 374bp fragment corresponding to EDA+Fn detected as two separate bands after the agarose gel electrophoresis.

The primer sequence for EDA+/-Fn was
5'GGAGAGAGTCAGCCTCTGGTTCAG3' Forward,
5'TGTCCACTGGGCGCTCAGGCTTGTG3' Reverse.

PCR steps were as: initial denaturation for 5min at 95°C and 25 cycles of 30sec at 95°C for, annealing temperature for 30sec at 56°C, 30sec at 68°C and final extension for 5min at 68°C. The PCR product is separated by 1.5% agarose gel electrophoresis and visualised with ethidium bromide staining under UV light. The separated bands of EDA+/-Fn in the agarose gel were captured by ImageQuant 300 imager and ImageQuant Capture software (v1.0.0.4; GE Healthcare Life Sciences). Quantification of band density was done by Image count TL software (v1.0.0.4; GE Healthcare Life Sciences).

The 1:10 diluted cDNA synthesised after RNA extraction and reverse transcription PCR was used to study gene expressions by real-time PCR. Real-time PCR was performed by using TaqMan custom made FAM/MGB probes labelled TaqMan gene expression assay supplied by Applied Biosystems (Foster City, CA, USA). Each assay contains pre-formulated primers and TaqMan FAM/MGB probes in a 20x concentration and was supplied for 250 reactions at a 20µl reaction volume. Pre-optimized TaqMan gene expression assays containing FAM/MGB dye-labeled probes were used for the expression analysis of target gene of interest. TaqMan endogenous control for GAPDH gene as custom made primer and probe sets labelled with VIC/MGB reporter dye was used as a housekeeping gene to normalise the gene expression supplied by Applied Biosystems. To present the relative gene expression, the widely used comparative C_T method also referred to as the $2^{-\Delta\Delta C}$ method was used.

Statistical Analysis

Data handling, Statistical analysis and presentation were performed using Microsoft Excel 2010 (Microsoft Corporation) and GraphPad Prism, v4.0 (GraphPad Software, Inc). Results were expressed as Mean ± SEM (standard error of the mean) of 3-6 independent experiments. Comparison between the means of 2 groups was made by Student's t-test. Comparison between the means of more than two groups was made by Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test. P value <0.05 was considered as significant.

Results

The effect of CCN3 on TGFβ1-mediated signalling pathways in human podocyte culture

Podocytes were allowed to differentiate for 14 days. Overnight serum starved podocytes were treated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml and compared with vehicle (0.1% w/v BSA) for 1h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for phosphorylation of pSmad1/5/8, pSmad2, pSmad3, pP38 and tubulin as housekeeping protein. TGFβ1 significantly induced the pSmad1/5/8, pSmad2, pSmad3 and pP38 phosphorylation as compared to vehicle. CCN3 significantly downregulated the TGFβ1-induced pSmad1/5/8 phosphorylation, however, did not alter the phosphorylation of other protein (Figure 1 and Figure 2).

Effect of CCN3 on TGFβ1-mediated alternative splicing of EDA+Fn in human podocyte culture

Podocytes were grown on 6 well cell culture plate and allowed to differentiate for 14 days and serum

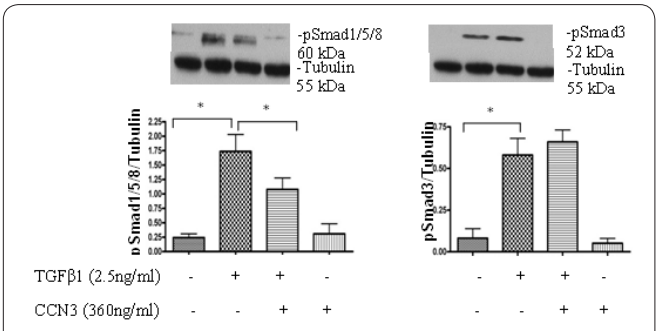

Figure 1. The pSmad1/5/8 and pSmad3 protein phosphorylation indexed to tubulin as housekeeping protein. Podocytes were treated with TGFβ1 2.5 ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml and compared with vehicle (0.1 % w/v BSA) for 1h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

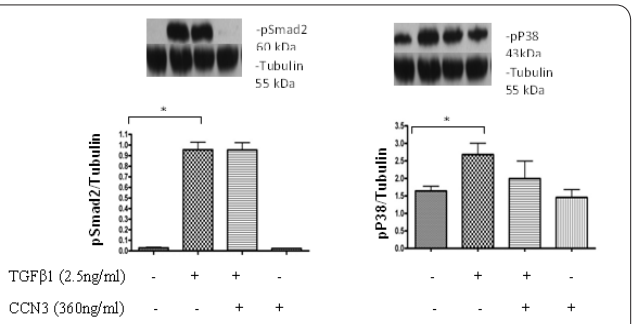

Figure 2. The pSmad2 and pP38 protein phosphorylation indexed to tubulin as housekeeping protein. Podocytes were treated with TGFβ1 2.5 ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml and compared with vehicle (0.1 % w/v BSA) for 1h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

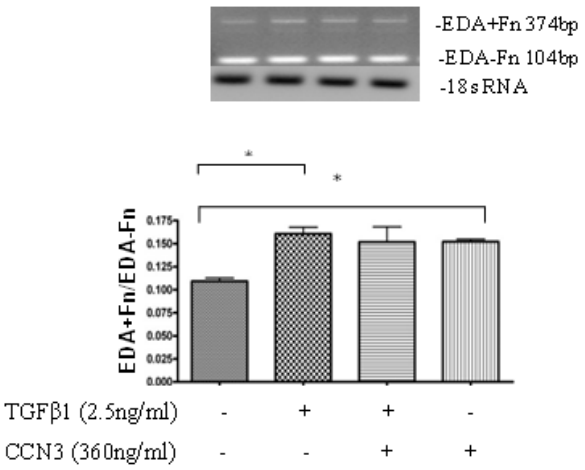

Figure 3. EDA+/- mRNA expression demonstrated by RT-PCR in human podocytes culture. Podocytes were treated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml and compared with vehicle (0.1% w/v BSA) for 24h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

starved overnight. Podocytes were co-incubated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml and compared with vehicle (0.1% w/v BSA) for 24h. Cells were lysed and RNA was extracted. RT-PCR was performed to look for EDA+Fn to EDA-Fn mRNA ratio. TGFβ1 significantly increased EDA+/-Fn mRNA ratio as compared to vehicle. CCN3 did not alter the TGFβ1-increased EDA+/- Fn mRNA ratio. CCN3 alone significantly increased the EDA+/-Fn mRNA ratio (Figure 3).

Effect of CCN3 on TGFβ1-mediated protein expression of EDA+Fn in human podocyte culture

Podocytes were allowed to differentiate, serum starved overnight and treated with TGFβ1 1.25ng/ml, TGFβ1 1.25ng/ml and CCN3 360ng/ml, TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml and compared with vehicle (0.1% w/v BSA) for 72h. Cells were lysed and protein extraction was done. Western blot analysis was performed to look for

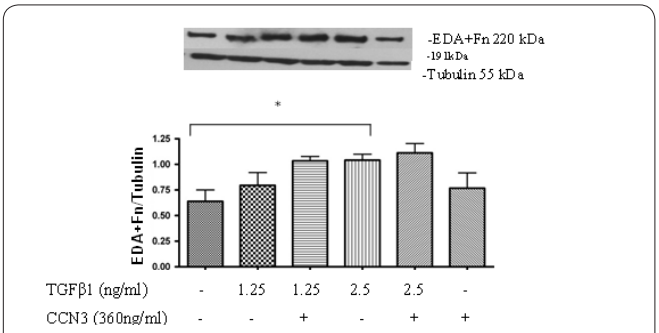

Figure 4. EDA+Fn protein expressions indexed to tubulin as housekeeping protein. Podocytes were treated with TGFβ1 1.25ng/ml, TGFβ1 2.5ng/ml, co-incubated with CCN3 360ng/ml and compared with vehicle (0.1 % w/v BSA) for 72h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

expression of EDA+Fn and tubulin as housekeeping protein. TGFβ1 2.5ng/ml significantly induced the EDA+Fn expression as compared to vehicle, however, CCN3 did not alter the TGFβ1-induced EDA+Fn expression (Figure 4).

Effect of CCN3 on TGFβ1-mediated gene expression of ECM proteins in human podocyte culture

Podocytes were allowed to differentiate, serum starved overnight and treated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml, compared with vehicle (0.1 % w/v BSA) for 72h. Cells were lysed and RNA was extracted. Real Time-PCR was performed to look for the gene expressions of Fn, collagen I, collagen IV and GAPDH as housekeeping gene. TGFβ1 significantly induced the gene expressions of Fn, collagen I and collagen IV normalised to GAPDH gene expressions as compared to vehicle. CCN3 did not alter the TGFβ1-induced gene expressions of Fn, collagen I, collagen IV, however, CCN3 alone induced the gene expressions of Fn, collagen I, collagen IV normalised to GAPDH gene expressions as compared to vehicle (Figure 5).

Discussion

In these studies, we have examined the effect of CCN3 on TGFβ1 mediated signalling, regulation of alternative splicing and expression of EDA+Fn and other extra cellular matrices (ECM) proteins gene expressions

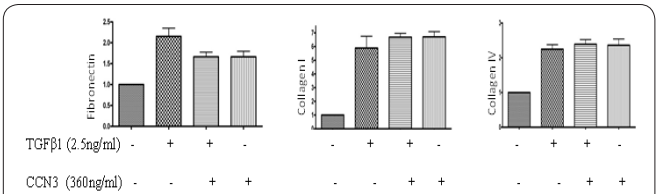

Figure 5. The gene expressions of Fn, Collagen I, Collagen IV normalised to GAPDH gene expression. Podocytes were pre-incubated (1h) and co-incubated with TGFβ1 2.5ng/ml, TGFβ1 2.5ng/ml and CCN3 360ng/ml, CCN3 360ng/ml compared with vehicle (0.1% w/v BSA) for 24h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

in human podocytes culture.

The results show that TGFβ1 significantly induced the phosphorylation of pSmad1/5/8, pSmad2, pSmad3 and pP38 as compared to vehicle. CCN3 significantly downregulated the TGFβ1-induced phosphorylation of pSmad1/5/8, however, did not alter the phosphorylation of other proteins. These results describe a novel function of CCN3 in regulation of TGFβ1 mediated non-canonical Smad signalling in human podocyte culture.

In addition to above results, TGFβ1 significantly induces the alternative splicing and protein expression of EDA+Fn. TGFβ1 significantly induces gene expressions of Fn, collagen I, collagen IV. However, CCN3 did not alter the TGFβ1 induced alternative splicing and protein expression of EDA+Fn. CCN3 also did not alter the TGFβ1-induced gene expressions of Fn, collagen I, collagen IV. These results suggest that TGFβ1 mediated non-canonical Smad signalling was not involved in regulation of alternative splicing, expression of EDA+Fn protein and gene expressions of Fn, collagen I, collagen IV in human podocytes culture.

However, CCN3 alone significantly induced the EDA+/-Fn mRNA ratio. CCN3 also induced the gene expression of ECM proteins Fn, Collagen I and Collagen IV. These results suggest that possible critical role of CCN3 in the regulation of alternative splicing of EDA+Fn and ECM proteins gene expression in human podocyte culture through some other mechanism.

This is the first evidence of CCN3 mediated downregulation of the TGFβ1 mediated non-canonical Smad signalling in human podocytes and in any cell type hence is very important and significant result in TGFβ1 mediated signalling with CCN3.

CCN3 also known as IGFBP- rP3 and as it shows similarities with Insulin-like growth factor binding protein, von Willebrand factor, and thrombospondin 1. Its C-terminal module contains cysteine knot motif which facilitates heterodimerization of different growth factors and matrix proteins (1). Thus this could be the possible mechanism of CCN3 mediated effect in human podocytes in culture.

Recently CCN3 has been shown to antagonise the activity of CTGF (1). CTGF functions downstream of TGFβ1 which amplifies the effects of TGFβ1 and increases the ECM production and fibrosis (13). It has been shown that elevated level of TGFβ1 induces the overexpression of CTGF (14). It has been shown that TGFβ1 induced Smad1 is CTGF dependent (15). CTGF demonstrated to increases TGFβ1 mediated deposition of Fn in ECM (4,5). It has been shown that in diabetic nephropathy CTGF was expressed in podocytes (17). Thus another possible mechanism could be the CCN3 mediated downregulation of CTGF which in turn regulate the TGFβ1 mediated non-canonical Smad signalling pathway. CCN3 has been shown to interact with cell surface integrins receptors (8). Thus CCN3 interaction with its own receptors could be another possible mechanism in CCN3 mediated events in human podocytes culture. CCN3 has been shown to bind BMP2 and regulates TGFβ1 non-canonical smad signalling and thus could be another possible mechanism of CCN3 mediated events in human podocytes culture (16).

This is the first evidence of the downregulation of TGFβ-mediated activation of a Smad1/5/8 signalling pathway by CCN3 in any cell type. Targeting CCN3 mediated events could provide exciting outcomes in the understanding of molecular mechanism of TGFβ events in fibrosis.

Abbreviations

ALK-Activin-like kinases, ANOVA-Analysis of Variance, BCA-Bicinchoninic acid, BSA- Bovine serum albumin, BMP- Bone marphogenic proteins, CTGF-connective tissue growth factor, CKD-Chronic kidney disease, cDNA- complementary deoxyribonucleic acid, , DN-Diabetic nephropathy, DNA-Deoxyribonucleic acid, ECM-extra cellular matrix, EDA- Extra domain A, EDB-Extra domain B, EMT- Epithelial-Mesenchymal transition, EDTA-Ethylenediaminetetraacetic acid, FSGS-Focal segmental glomerulosclerosis, FCS-Fetal calf serum, Fn-Fibronectin, GFB-Glomerular filtration barrier, GFs-Growth factors, PCR-polymerase chain reaction, PAGE-Polyacrylamide gel electrophoresis, PBS-Phosphate-buffered saline, PTEC- Primary tubular epithelial cells, RNA-Ribonucleic acid, RNPs-Ribonucleoproteins, RT-PCR-Reverse transcriptase-Polymerase chain reaction, RPMI- Rosewell Park Memorial Institute, RGD-Arg–Gly–Asp, SD-slit diaphragm, SDS-Sodium dodecyl sulphate, TGFβ – Transforming growth factorβ, VEGF- Vascular endothelial growth factor.

Declarations

Ethics approval and consent to participate- Not applicable.

Consent for publication- Not applicable.

Availability of data and materials- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests- The authors declare that they have no competing interests.

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Authors' contributions-

Dr Tarunkumar H Madne- All research works carried by this author.

Dr Mark Edward Carl Dockrell- Lead supervisor.

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