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A 3D tri-culture system reveals that activin receptor-like kinase 5 and connective tissue growth factor drive human glomerulosclerosis

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Abstract

Glomerular scarring, known as glomerulosclerosis, occurs in many chronic kidney diseases and involves interaction between glomerular endothelial cells (GEC), podocytes and mesangial cells (MCs), leading to signals that promote extracellular matrix deposition and endothelial cell dysfunction and loss. We describe a 3D tri-culture system to model human glomerulosclerosis. In 3D monoculture, each cell type alters its phenotype in response to TGF β , which has been implicated as an important mediator of glomerulosclerosis. GECs form a lumenized vascular network, which regresses in response to TGF β . MCs respond to TGF β by forming glomerulosclerotic-like nodules with matrix deposition. TGF β treatment of podocytes does not alter cell morphology, but increases connective tissue growth factor (CTGF) expression. BMP7 prevents TGF β -induced GEC network regression, whereas TGF β -induced MC nodule formation is prevented by SMAD3 siRNA knock down or ALK5 inhibitors, but not BMP7, and increased phospho-SMAD3 was observed in human glomerulosclerosis. In 3D tri-culture, GECs, podocytes and MCs form a vascular network in which GECs and podocytes interact intimately within a matrix containing MCs. TGF β treatment induces formation of nodules, but combined inhibition of ALK5 and CTGF is required to prevent TGF β -induced nodule formation in tri-cellular cultures. Identification of therapeutic targets for glomerulosclerosis depends on the 3D culture of all three glomerular cells.

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Author contributions

JPW, YCR, PDU contributed to conception and design of the study and acquisition and analysis of data. JNS contributed to acquisition and analysis of data. NWM, JSP and JRB contributed to conception and design, and analysis of data. All authors contributed to drafting and revising the article, and have approved the final version.

Conflicts of interest

None of the authors have any conflicts of interest to declare

Keywords

glomerulosclerosis; endothelial; mesangial; podocyte; TGF-beta

Introduction

Glomerulosclerosis, characterised by fibrotic glomerular scarring with glomerular endothelial cell (GEC) dysfunction and loss, occurs in many primary renal and systemic chronic kidney diseases, including idiopathic focal segmental glomerulosclerosis and the nephropathy that commonly accompanies diabetes. Progressive chronic kidney disease (CKD) leads to end-stage renal failure (ESRF), which carries a high morbidity and mortality. In the US, the prevalence of CKD over the age of 60 is 33.2%, and by the end of 2013, nearly half a million people were undergoing dialysis for ESRF (1). Dialysis provides only 10–15% of normal renal function, and carries a greater risk of death compared to the normal population (2). Transplantation is not available to all, and carries risks associated with immunosuppression; the current average lifespan of a renal graft is 10–15 years (3). Preventing or reversing glomerulosclerosis would dramatically improve the health and life expectancy of a large population of patients, and provide a major financial saving to healthcare providers. However, at present the mechanisms of glomerulosclerosis are poorly understood and therapeutic strategies are limited.

There are numerous animal models of glomerulosclerosis (4), but these use a range of insults that often do not reflect the aetiology in human disease, take weeks or months to evolve, and have been poor predictors of therapeutic responses in humans. Human cell culture systems represent an alternative approach, but to date they have been of limited value in modelling glomerulosclerosis because the development of lesions is dependent on complex cellular networks and matrix interactions that do not occur in typical cell culture models, often involving a single cell type in monolayer (2D) culture.

TGF β has been implicated as an important mediator of glomerulosclerosis, and a potential therapeutic target for preventing the fibrotic changes that accompany many glomerular diseases. TGF β is increased in the glomeruli of patients with glomerulosclerosis associated with a range of chronic kidney diseases and chronic allograft nephropathy (5, 6, 7). Animal studies have shown a causative effect for TGF β in glomerulosclerosis. A transgenic mouse with increased plasma levels of TGF β develops glomerular scarring typical of chronic kidney disease (8, 9), and *in vivo* transfection of TGF β into rat kidney induces glomerulosclerosis with extracellular matrix expansion and proteinuria (10). Furthermore, increased expression of TGF β mRNA and protein is seen in glomerular cells in rats after induction of diabetes with streptozotocin (11), and heterozygosity for TGF β type II receptor attenuates the degree of mesangial expansion after streptozotocin treatment (12). Evidence from animal models indicates that BMP7 may act as an anti-fibrotic agent, counteracting the effects of TGF β , particularly the tubulointerstitial compartment (13). This has not yet translated into clinical trials, and whether BMP7 can ameliorate the glomerulosclerotic component of fibrosis in human kidneys is not known. Models to assess the effects of BMP7 in human glomerulosclerosis are lacking.

The glomerular filtration unit is a multicellular structure, consisting of a complex basement membrane partitioning glomerular endothelial cell (GEC)-lined capillaries and mesangial cells (MCs) on the side facing the blood from podocytes that face into Bowman's space (14). Regardless of the aetiology, glomerulosclerosis is characterised by expansion of matrix within the mesangio-capillary compartment, initially "thickening" the basement membrane and eventually replacing the glomerulus entirely (15). To capture this complexity, we have developed a three-dimensional *in vitro* human glomerular cell culture model of glomerulosclerosis. We first used the 3D system to culture each of the resident human glomerular cells in isolation, and then refined the system to form an *in vitro* human glomerular vascular structure through 3D "tri-culture" of human GECs, MCs and podocytes. We demonstrate that TGF β induces a glomerulosclerotic phenotype in this model, which can be used to screen therapeutic ligands such as BMP7. Although the signalling pathways can be studied in culture of individual cell types, the mechanism involves an interaction between all three types and identification of therapeutic targets depends on the 3D culture of all three glomerular cells.

Materials & Methods

2D cell monocultures

Human GECs were cultured in 500 ml of basal medium, 25 ml of FBS, 5 ml of endothelial cell growth supplement (ECGS) and 5 ml of penicillin/streptomycin solution. Human MCs were cultured in 500 ml basal medium, 10 ml of fetal bovine serum (FBS), 5 ml of mesangial cell growth supplement, and 5 ml of penicillin/streptomycin solution (GECs, MCs, media and supplements were all from ScienCell Research Laboratories, San Diego, CA. Primary human podocytes (Celprogen, Torrance, CA) were cultured in the supplier's medium with serum. All were used between passages 2 and 6. Phenotypic characterisation showed that GECs express CD31 and von Willebrand factor, MCs express smooth muscle actin, and podocytes express ezrin, WT1, synaptopodin, NPHS2 and nephrin and form foot processes and slit diaphragms (supplementary material, Figure S1).

Formation of 3D culture

GECs, MCs and podocytes in monoculture or co-culture were suspended in a solution of rat tail type I collagen (1.5 mg/ml; BD, NJ, USA) and human plasma fibronectin (90 μ g/ml; Millipore, MA, USA) in 25 mM HEPES and 1.5 mg/ml NaHCO₃-buffered M199 medium at 4°C, and the pH was adjusted to 7.4 using 0.1 M HCl. The suspension was pipetted into 48-well plates (320 μ l per well), with 5×10^5 GECs per well (monoculture), 2.5×10^5 podocytes per well (monoculture) and 5×10^5 MCs per well (monoculture). After the matrix had polymerised, 0.5 ml of media was pipetted onto the gel. For co-cultures, 330,000–340,000 GECs, 50,000–70,000 podocytes and 20,000–24,000 MCs were added per well (a ratio of 16:3:1) and triculture medium was used (consisting of 95% RPMI 1640 (Gibco™ by Thermo Fisher, UK), 2% FBS, 1% penicillin/streptomycin, 1% ITS mix (insulin 1 mg/ml, apo-transferrin 1 mg/ml, sodium selenite 3.4 μ M; ScienCell Research Laboratories, San Diego, CA, catalogue no. 0803) and 1% endothelial cell growth supplement (ECGS; ScienCell Research Laboratories, catalogue no. 1052). For confocal microscopy, Ibidi μ -slides (Ibidi, Martinsried, Germany; catalogue no. 81506) were used with the same density

and ratio of cells. Concentrations for stimulation were 10 ng/ml TGF β (R&D Systems, MN, USA) or 100 ng/ml BMP7 (R&D Systems, MN, USA).

Lentivirus transduction of GECs

GECs were transduced with green fluorescent protein (GFP) using lentiviral vector containing the human polypeptide chain elongation factor-1 (EF-1) promoter by using 10 μ l virus in 4 ml media per approximately 300,000 GECs. Cells were counted and plated into 6-well TC treated plates (Corning Inc., Corning, NY, USA), left in culture 24 hours and then culture medium was replaced and cells were treated with lentivirus for 48 hours. After treatment, GECs were ready to be counted and incorporated into 3D culture.

Imaging of 3-dimensional cultures

Phase contrast and epifluorescence microscopy was performed on a Leica DMI3000B manually inverted microscope with ImagePro software (MediaCybernetics, MD, USA). Confocal microscopy was performed using a Leica TCSSP5 microscope with Leica Application Suite software (Wetzlar, Germany). GECs were either transduced with GFP as above or stained with *Ulex europeus* agglutinin I (Vector Laboratories, CA, USA). Podocytes were labelled with PKH26 dye (Sigma-Aldrich, MO, USA) before implantation into the matrix, using the manufacturer's protocol. MCs were labelled with Celltracker™ Blue CMAC dye before implantation into the matrix, using the manufacturer's protocol (Life Technologies, Thermo Fisher Scientific, MA, USA).

For histology, 3D culture matrices were embedded in HistoGel (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions, fixed in 4% paraformaldehyde overnight, paraffin wax-embedded for sectioning, and stained with hematoxylin and eosin (H&E).

For scanning electron microscopy, 3D matrices were washed in normal saline, fixed in 2% glutaraldehyde in 0.1 M PIPES, pH 7.4, in 0.1 M sodium cacodylate buffer for 14 hours, dehydrated with increasing ethanol concentrations to 100%, frozen in liquid nitrogen, fractured with a razor blade and hammer, critical point dried, mounted on Cambridge SEM stubs with silver DAG (Agar Scientific, Stansted, UK, catalogue no. AGG3648), sputter-coated with 10 nm gold and viewed with a FEI XL30 FEG scanning electron microscope operated at 5 keV (FEI, Oregon, USA).

Staining of human collagen in MC 3D cultures

After culture, matrices were embedded in HistoGel, fixed in 4% paraformaldehyde and paraffin wax-embedded for sectioning and staining for human collagen type IV (mouse anti-human collagen IV monoclonal antibody 2150–0121, Bio-Rad, CA, USA) and human collagen type I/III (rabbit anti-human collagen I/III polyclonal antibody 2150–2210, Bio-Rad, CA, USA). After automated antigen retrieval processing (PT Link Machine, DAKO, Denmark), sections were washed three times for five minutes in wash buffer (EnVision Flex Wash Buffer, DAKO, Denmark), blocked for ten minutes in blocking reagent (EnVision FLEX peroxidase-blocking reagent, DAKO, Denmark), washed a further three times, incubated with primary antibody diluted in substrate buffer (EnVision Flex Substrate Buffer,

DAKO, Denmark) for one hour at room temperature, washed three times, and incubated with secondary antibody (EnVision FLEX/HRP, DAKO, Denmark) for 30 minutes at room temperature. After three washes, 3,3-diaminobenzidine tetrahydrochloride (DAB) solution (EnVision FLEX DAB, DAKO, Denmark) was added with H&E counterstain. Acellular matrix (consisting of rat type I collagen) was used as a negative control, and normal human kidney was used as a positive control.

SMAD2 and SMAD3 siRNA knockdown of mesangial cells

A 20 μ M solution of lyophilised siRNA in siRNA buffer (5 $\times$, Dharmacon, CO, USA) was used for knockdown of MCs. MCs plated on day 1 at 150,000 per well (in supplied medium consisting of 500 ml of basal medium, 10 ml of FBS, 5 ml of mesangial cell growth supplement) were transfected on day 3. Wells were first washed with 2 ml per well of Optimem 1 (Invitrogen, CA, USA) for two hours and then changed to fresh Optimem (1.6 ml per well). Four tubes containing DharmaFECT 1, a non-targeting control pool (siCP), ON-TARGET^{Plus}™ siGENOME™ Smartpool siSMAD2 and siSMAD3 were made up as per manufacturer's instructions (Invitrogen, CA, USA). Lipoplexes were left on the cells for 4 hours, and then removed; normal growth medium was added and left overnight before stimulation assays. For SMAD3 knockdown, the same method was also carried out using DharmaFECT 2 and 4. Knockdown was confirmed by immunoblotting for total SMAD2 and total SMAD3.

ALK5 inhibition and CTGF neutralising antibody in 3D matrices

For inhibition of ALK5 in 3D, 616456 compound (Millipore, MA, USA) was added to the 0.5 ml of media on top of the 3D matrices, at a concentration of 2 μ M in the presence of TGF β at 10 ng/ml. For neutralisation of CTGF, CTGF neutralising antibody (ab109606, Abcam, Cambridge, UK) was used at a concentration of 8 μ g/ml.

Immunoblotting

GECs or MCs were grown in 6-cm dishes to ~80% confluence and treated with 10 ng/ml TGF β (R&D Systems, MN, USA) and/or 100 ng/ml BMP7 (R&D Systems, MN, USA) in mesangial cell or endothelial cell medium as described above. At 6 hours the medium was aspirated and the cells washed with ice cold PBS, which was then aspirated. The dishes were then placed on ice and the cells scraped into 350 μ l RIPA buffer (ThermoFisher Scientific, MA, USA) with protease inhibitors (Roche, Switzerland), transferred to an Eppendorf tube and agitated on ice three times for ten seconds every two minutes, and centrifuged at 1200 rpm for 20 minutes at 4°C. The supernatant was aspirated to another Eppendorf tube for storage at -80°C. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, MA, USA). Proteins (15 μ g) in Laemmli sample buffer were separated by SDS-polyacrylamide gel (10%) electrophoresis and then transferred to polyvinylidene fluoride membrane. Blots were blocked in 5% dried milk powder plus 0.05% Tween 20 in PBS for one hour at room temperature or overnight at 4°C. After blocking, they were immunoblotted with rabbit anti-pSMAD2 (3108, Cell Signalling, MA, USA), rabbit anti-pSMAD1/5 (9516, Cell Signalling, MA, USA), rabbit anti-pSMAD3 (1880-1, Epitomics, Abcam, Cambridge, UK), rabbit anti-tSMAD2 (3122, Cell Signalling, MA, USA), rabbit anti-tSMAD3 (9513, Cell Signalling, MA, USA), or mouse anti- β actin

(A5441, Sigma-Aldrich, MO, USA) for two hours (40 minutes for actin) at room temperature or overnight at 4°C. This was followed by incubation for two hours at room temperature with anti-mouse (1:5000, DAKO, Denmark) or anti-rabbit IgG horseradish peroxidase conjugate (1:4000, DAKO, Denmark) and detection by enhanced chemiluminescence according to the manufacturer's instructions (West Pico for β actin and West Dura for SMADs, Thermo Fisher Scientific, MA, USA).

Quantitative reverse transcriptase PCR analysis (qPCR)

RNA was isolated from 3D matrices by Trizol extraction. 1 ml of Trizol was added per 300 μ l of matrix and vortexed for 15 s, left at room temperature for 15 minutes, and vortexed for a further 15 s. Chloroform (0.2 ml per 1 ml of Trizol) was added, shaken vigorously for 5 s, left to stand for 3 minutes at room temperature, and then centrifuged at 12,000 g at 4°C for 15 minutes. A half volume of 100% ethanol was added to the RNA (aqueous phase), which was centrifuged at 8000 rpm for 1 minute at 4°C using the MoBio RNA Isolation kit (MoBio, CA, USA), washed with 500 μ l of guanidinium thiocyanate aqueous lysis solution (Mo Bio, CA, USA, catalogue No. 15000–1), and centrifuged at 8000 rpm for 1 minute at 4°C. 45 μ l of Tris-HCl (pH 7.5), sodium chloride and magnesium chloride solution and 5 μ l of DNase was added to the filter and incubated at room temperature for 15 minutes. 400 μ l of aqueous solution of guanidine thiocyanate was then added and centrifuged at 8000 rpm for 1 minute at 4°C. This was repeated twice with 500 μ l of ethanol solution prior to centrifugation at 10,000 rpm for 2 minutes at 4°C. The precipitate was washed twice with aqueous ethanol (Mo Bio, CA, USA, catalogue No. 15000–4), centrifuging at 8000 rpm for 1 minute at 4°C each time, and then centrifuged at 10,000 rpm for 2 minutes at 4°C. Finally it was eluted with 30 μ l of RNase-free water, incubated for 2 minutes and centrifuged at 8000 rpm for 1 minute at 4°C. DNase-digested total RNA (300–700 ng) was reverse transcribed using a high capacity cDNA reverse transcription kit (Mo Bio, CA, USA, catalogue no. 15100–50) as described in the manufacturer's instructions. qPCR reactions were prepared with 45 ng of cDNA using the SYBR® Green Jumpstart™ Taq Readymix™ (Sigma-Aldrich, MO, USA) containing 200 nM of the relevant sense and antisense primers and 10 nM fluorescein (Invitrogen, NY, USA). Reactions were amplified on an iCycler (Bio-Rad, CA, USA) using Quantitect Primers for *COL1A1*, *COL4A1*, *CTGF*, *MMP2*, *TGFBR2*, *ALK1*, *ALK5*, *ALK3*, *ALK6* and *ALK2* (Qiagen, Germany). The relative expression of target mRNAs was normalized to the housekeeping genes *B2M* or *GAPDH* using the C_t method and expressed as the fold-change relative to the control.

Effect of TGF β on MCs on 2D coated surfaces

MCs were plated onto non-treated plastic, culture media-treated plastic, or plastic coated by incubation overnight at 37°C with 1.5 mg/ml type I rat tail collagen (BD, Franklin Lakes, N.J., USA) in PBS, or 0.1 mg/ml human fibronectin (Millipore, Billerica, Mass., USA) in PBS. MCs were cultured in 6-cm culture dishes (Corning, NY, USA) at low density. Cells were cultured in 3 ml of media with or without 10 ng/ml of TGF β , which was changed every 24 hours. Cultures were followed by phase contrast microscopy using a Leica DMI 3000B manually inverted microscope until three days after confluence.

Human kidney tissue staining

Kidney tissue was obtained with Health Research Authority approval (Cambridge University Hospitals, reference 07/QO108/49) from subjects with diabetic nephropathy (n=3) or focal segmental glomerulosclerosis (n=3) and from the histologically normal poles of kidneys removed because of tumours (n=6). The tissue was fixed in 4% paraformaldehyde, paraffin wax-embedded for sectioning, and stained with rabbit anti-pSMAD2 (3108, Cell Signalling, MA, USA), rabbit anti-pSMAD3 (1880-1, Epitomics, Abcam, Cambridge, UK), and rabbit anti-pSMAD1 (9553, Cell Signalling, MA, USA). After automated antigen retrieval processing (PT Link Machine, DAKO, Denmark), the sections were washed in EnVision Flex Wash Buffer (DAKO, Denmark) for five minutes three times, blocked for ten minutes in EnVision FLEX Peroxidase-blocking reagent (DAKO, Denmark), then washed in wash buffer again for five minutes three times. Sections were incubated in the relevant collagen primary antibody diluted in EnVision Flex Substrate Buffer (DAKO, Denmark) for one hour at room temperature and then washed for five minutes three times in wash buffer. Sections were incubated with secondary antibody (EnVision FLEX/HRP, DAKO, Denmark) for 30 minutes at room temperature, after which time three five minute washes were performed with wash buffer. 3,3-diaminobenzidine tetrahydrochloride (DAB) solution (EnVision FLEX DAB, DAKO, Denmark) was added to visualise the antibody, and sections were counterstained with H&E. Human lung tissue was used as a positive control.

Quantitative image analysis and statistical tests

Quantification of cord networks and nodule counts within the 3-D matrix was performed by taking random low-magnification images through the matrix with phase contrast or fluorescence microscopy, using an Axiovert 200 M Carl Zeiss microscope or Leica DMI 3000B manually inverted microscope. Images were analysed using Image J software (NIH, USA) to quantify cord length (total and average length), branching points (nodes), tube width and cord number as well as MC nodule number. Differences between treatments were analysed using an unpaired two-tailed Student t test. Experiments were replicated at least three times.

Results

GECs, MCs and podocytes in 3-dimensional culture form a vascular glomerular structure

Human GECs cultured in two dimensions on tissue culture-treated plastic form a confluent monolayer with a cobblestone appearance (data not shown). Cultured in the 3D matrix, GECs form networks of cords surviving up to 6 days (Fig. 1a). Narrow lumens can be detected by 24 hours, and appear to form by coalescence of intracellular vacuoles (Fig. 1a inset and 1b); potential fenestrae can be identified by electron microscopy (Fig. 1c). MCs cultured within the 3D system randomly disperse throughout the matrix and associate intimately with collagen fibres (Fig. 1d). Podocytes cultured within the 3D system maintained characteristic morphological features, including foot processes visible on scanning electron microscopy (Fig. 1e,f).

Optimisation of the media and cell ratios allowed tri-culture of the three glomerular cell types in 3D (Fig. 2a, b). GECs formed lumenised cords, with which podocytes align within

24 h, maintaining foot processes that extend around the GECs (Fig. 2c, 2d, 2e). MCs surrounded these networks, but were not intimately associated with either the GECs or podocytes.

TGF β causes phenotypic changes in GECs, MCs and podocytes in 3D monoculture, which BMP7 prevents in ECs

In 3D monoculture of GECs, TGF β induces a loss of arborisation of vascular networks (Fig. 3a) consistent with capillary rarification seen in glomerulosclerosis. Co-treatment with BMP7 prevents this TGF β -induced decrease in arborisation (Fig. 3a). Podocytes cultured in 3D did not alter their morphology in response to TGF β ; however, TGF β increased connective tissue growth factor (CTGF) mRNA, and this effect was more marked in 3D culture compared to 2D (Fig. 3b).

MCs cultured in 2D on plastic coated with or without rat tail collagen type I or fibronectin grow to confluence and do not alter their morphology in response to TGF β (supplementary material, Figure S2). In 3D MC cultures, TGF β treatment promotes formation of large nodules within 24 hours, which contain both cells and matrix proteins (Fig. 4b, e, f). Human collagen type I and IV mRNAs were also increased in the TGF β -treated group (Fig. 4f), and human collagen type I/III (the antibody used recognizes both collagen type I & III) (Fig. 4c) & human collagen type IV protein (Fig. 4d) were located within and around the nodules; human collagen was not detected in non-nodular areas or within the rat tail type I collagen matrix scaffold (data not shown). BMP7 did not modulate TGF β -mediated collagen type I and IV mRNA expression or nodule formation (Fig. 4f).

BMP7 modulates TGF β SMAD responses in GECs but not MCs

In view of the different effects of BMP7 on TGF β -induced GEC network loss and TGF β -induced MC nodule formation, we assessed SMAD responses in the two different cell types in response to treatment with TGF β , with or without BMP7. In GECs, TGF β led to phosphorylation of both SMAD2 and SMAD3. BMP7 prevented TGF β -induced SMAD2 phosphorylation, but not SMAD3 phosphorylation (supplementary material, Figure S3a, c), and BMP7 induced phosphorylation of SMAD1/5, which was not prevented by TGF β .

In MCs, BMP7 did not prevent TGF β -induced SMAD2 or SMAD3 phosphorylation (supplementary material, Figure S3b, c), while TGF β did decrease BMP7-induced SMAD1/5 phosphorylation. In other words, SMAD phosphorylation in responses to TGF β and BMP7 differs between GECs and MCs.

ALK5 inhibition prevents TGF β -induced nodule formation in MC 3D monoculture

In 3D MC cultures, TGF β treatment increased ALK5 mRNA and decreased ALK1 mRNA (Fig. 5a). This is likely to decrease TGF β RII/ALK1/ALK5 trimerisation and increase TGF β RII/ALK5 dimerisation, thereby increasing SMAD2/3 phosphorylation, and decreasing SMAD1/5 phosphorylation. These changes in receptor mRNA expression were not prevented by co-treatment with BMP7 (Fig. 5a). An ALK5 inhibitor decreased TGF β -induced nodule formation (Figure 4g). *SMAD2* siRNA reduced SMAD2 protein expression but did not alter the number of nodules formed (Fig. 4h), whereas *SMAD3* siRNA reduced

SMAD3 protein expression and prevented TGF β -induced nodule formation (Fig. 4h). This is consistent with an increase in phospho-SMAD3, which we have also observed in human glomerulosclerosis associated with diabetic nephropathy and focal segmental glomerulosclerosis. (Fig. 5b).

Prevention of a TGF β -induced glomerulosclerotic phenotype in 3D triculture of glomerular cells requires combined ALK5 inhibition and CTGF neutralisation

TGF β treatment of GEC, MC and podocyte 3D tri-cultures induced nodule formation within 24 hours (Fig. 6a,b), reduced interaction between GECs and podocytes with fewer podocytes within the nodule areas (Fig 6c), caused loss of GEC network arborisation (Fig. 6b,c), and increased collagen I and IV expression (Fig. 6d). These changes are characteristic of glomerulosclerosis. BMP7 co-treatment did not alter this phenotype (Fig. 6d). ALK5 inhibition was less effective at reducing TGF β -induced nodule formation in tri-culture than in MC monoculture. However, combined treatment of the tri-cultures with a CTGF-neutralising antibody and ALK5 inhibitor prevented TGF β -induced nodule formation (Fig. 6e). ALK5 inhibition reduced the increased collagen I and IV expression (Fig. 6e).

Discussion

The 3D culture of human glomerular cells described here reveals their ability to assemble into a glomerular vascular structure, when cultured within a matrix at optimised ratios. In this structure, GECs form vessels with lumens and potential fenestrae, which associate intimately with podocytes in a matrix containing MCs. TGF β induces a phenotype characteristic of glomerulosclerosis, with loss of GEC vascular structures, podocyte detachment, nodule formation and matrix deposition. These changes provide quantifiable measures that can be used to assess signalling targets, which can involve cross-talk between cells and therefore cannot be assessed in traditional 2D monolayer cultures.

BMP7 prevented the GEC component of the glomerulosclerotic phenotype, but not the MC component. This may explain the less impressive effects of treatment with BMP7 (or BMP7 ligand) on glomerulosclerosis compared to tubulointerstitial fibrosis in animal models. In murine models, renal fibrosis has been shown to be ameliorated by BMP7 (13). Our results suggest that in the glomerulus, different targets need to be modulated in different cell types, and BMP7 treatment alone will not be sufficient to ameliorate glomerulosclerosis. The results also suggest that downstream the TGF β and BMP7 pathways interact in different ways in different glomerular cell types. This was supported by the observation that in GECs, but not MCs, BMP7 influences TGF β -induced SMAD phosphorylation.

The ability of TGF β to modulate its own receptors has been previously described. For example, high concentrations of TGF β down-regulate TGF β RII and III receptors in human osteoblasts (16). Such changes are believed to form a feedback loop to dampen responses in the presence of high concentrations of TGF β (16). In 3D culture, TGF β increased the expression of ALK1 and decreased the expression of ALK5 in MCs. This was not preventable by BMP7. The response of a cell to TGF β is influenced by the heterodimeric or trimeric receptor formed to accept the ligand; TGF β associated with a heterodimer of TGF β RII and ALK5 signals through phosphorylation of SMAD2 and SMAD3, whereas

TGF β associated with a heterotrimer of TGF β RII, ALK1 and ALK5 signals through SMAD1, SMAD5 and SMAD8 (17). In the homeostatic state, this may provide a balanced response to TGF β , but an increase in ALK5 and decrease in ALK1 will tip this balance towards formation of the heterodimeric receptor and SMAD2/3 phosphorylation. This may exacerbate nodule formation by MCs, and provides the rationale for inhibiting ALK5 as a means of preventing nodule formation.

In 3D tri-culture, neither BMP7 administration nor ALK5 inhibition prevented nodule formation and collagen deposition. This is likely to be due to the effects of CTGF released from podocytes, and is consistent with the exacerbation of glomerular damage in transgenic mice with podocytes overexpressing CTGF in diabetes induced by streptozotocin (18). A combination of ALK5 inhibition and neutralising CTGF was needed to prevent the MC nodule formation in tri-culture.

In summary, we describe the first 3D tri-culture of human glomerular cells with formation of a glomerular vascular network. Although 3D culture of many cell types is well established, these are typically monocultures, which do not reflect the interactions between different cell types within a microenvironment. Organoid culture systems can address these issues, but defining the precise cellular composition and the manipulation of individual cell types is more challenging. In the system we describe, TGF β induces a phenotype characteristic of glomerulosclerosis. The signalling pathways can be studied in culture of individual cell types, but the mechanism involves an interaction between all three types. Identification of therapeutic targets may depend on the 3D model of all three glomerular cells described here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Collins AJ, Foley RN, Gilbertson DT et al. United States renal data system public health surveillance of chronic kidney disease and end-stage renal disease. *Kidney Int Suppl* 2015; 5: 2–7.
2. NHS Kidney Disease: Key Facts And Figures 2010.
3. Wolfe RA, Ashby VB, Milford EL et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med* 1999; 341:1725–1730. [PubMed: 10580071]
4. Yang HC, Zuo Y, and Fogo AB Models of chronic kidney disease. *Drug Discov Today Dis Models* 2010; 7:13–19. [PubMed: 21286234]
5. Shihab FS, Yamamoto T, Nast CC et al. Transforming growth factor-beta and matrix protein expression in acute and chronic rejection of human renal allografts. *J Am Soc Nephrol* 1995; 6:286–294. [PubMed: 7579098]
6. Yamamoto T, Noble NA, Cohen AH et al. Expression of transforming growth factor-beta isoforms in human glomerular diseases. *Kidney Int* 1996; 49:461–469. [PubMed: 8821830]
7. Iwano M, Kubo A, Nishino T et al. Quantification of glomerular TGF-beta 1 mRNA in patients with diabetes mellitus. *Kidney Int* 1996; 49:1120–1126. [PubMed: 8691733]

8. Sanderson N, Factor V, Nagy P et al. Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci U S A* 1995; 92:2572–2576. [PubMed: 7708687]
9. Kopp JB, Factor VM, Mozes M et al. Transgenic mice with increased plasma levels of TGF-beta 1 develop progressive renal disease. *Lab Invest* 1996; 74:991–1003. [PubMed: 8667617]
10. Isaka Y, Fujiwara Y, Ueda et al. Glomerulosclerosis induced by in vivo transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 1993; 92:2597–2601. [PubMed: 8254017]
11. Park IS, Kiyomoto H, Abboud SL et al. Expression of transforming growth factor-beta and type IV collagen in early streptozotocin-induced diabetes. *Diabetes* 1997; 46:473–480. [PubMed: 9032105]
12. Kim HW, Kim BC, Song CY et al. Heterozygous mice for TGF-betaIIIR gene are resistant to the progression of streptozotocin-induced diabetic nephropathy. *Kidney Int* 2004; 66:1859–1865. [PubMed: 15496156]
13. Wang S, Chen Q, Simon TC et al. Bone morphogenic protein-7 (BMP-7), a novel therapy for diabetic nephropathy. *Kidney Int* 2003; 63:2037–2049. [PubMed: 12753291]
14. Suh JH and Miner JH The glomerular basement membrane as a barrier to albumin. *Nat Rev Nephrol* 2013; 9:470–477. [PubMed: 23774818]
15. Schlöndorff D and Banas B The mesangial cell revisited: no cell is an island. *J Am Soc Nephrol* 2009; 20:1179–1187. [PubMed: 19470685]
16. Gebken J, Feydt A, Brinckmann J et al. Ligand-induced downregulation of receptors for TGF-beta in human osteoblast-like cells from adult donors. *J Endocrinol* 1999; 161:503–510. [PubMed: 10334821]
17. Upton PD and Morrell NW TGF-beta and BMPR-II pharmacology--implications for pulmonary vascular diseases. *Curr Opin Pharmacol* 2009; 9:274–280. [PubMed: 19321386]
18. Yokoi H, Mukoyama M, Mori K et al. Overexpression of connective tissue growth factor in podocytes worsens diabetic nephropathy in mice. *Kidney Int* 2008; 73:446–455. [PubMed: 18075496]

Figure 1. Human glomerular cell monocultures in 3D.

(A) ULEX staining of human GEC microvessel networks in the 3D matrix at $\times 40$ power. Inset: lumenisation of GEC cords revealed by GFP lentivirus-transduced GECs in triculture (MCs and podocytes not labelled), showing coalescence of vacuoles to form lumen. (B) Scanning electron microscopy (SEM) of GEC microvessel, showing the lumen (arrow) and (C) abluminal surface with fenestra (arrow). (D) SEM of a MC in the 3D matrix. (E) SEM of podocytes in the 3D matrix. (F) High magnification SEM view of podocyte foot process in the 3D matrix.

Figure 2. 3D tri-culture of human GECs, MCs and podocytes.

(A, B) Low and high magnification images of cultured GECs (labelled with ULEX-FITC), podocytes (labelled with PKH-Red) and MCs (labelled with CAMC-Blue) at 24 hours. (C, D) Association of GECs (ULEX-FITC) and podocytes (PKH-Red) in tri-cultures at 24 hours. (E) Scanning electron microscopy of GEC-podocyte interaction at 24 hours.

Figure 3. Responses to TGFβ of human glomerular endothelial cell microvessels and podocytes in 3D.

(A) Quantification of GEC network arborisation with TGFβ (10 ng/ml) or BMP7 (100 ng/ml) treatment and co-treatment, as assessed by cord number, total cord length, average cord length and branching points (n=3, standard deviation bars, 2-tailed Student's *t* test). (B) CTGF mRNA level in podocytes in 2D (black bars) and 3D (white bars) by quantitative reverse transcriptase PCR analysis in response to TGFβ (10 ng/ml) and BMP7 (100 ng/ml) stimulation and co-stimulation (fold change compared to control, n=3, standard deviation bars, 2-tailed Student's *t* test).

Figure 4. Mesangial nodule formation and matrix deposition in 3D cultures.

(A) MC 3D monoculture without the addition of TGFβ, and (B) in the presence of TGFβ, shown by H&E staining of paraffin-embedded sections. Human collagen type I/III (immunostained with an antibody that recognises both collagen type I and III) (C) and human collagen type IV (D) can be demonstrated in nodules by immunoperoxidase staining. (E) High-power view of MC nodule by scanning electron microscopy in cross section. (F) MC nodule count and collagen type I alpha1 (COL1a1), and collagen type IV alpha1 (COL4a1) RNA quantification (n=3). (G) Effect of ALK5 inhibition (Alk5i) on MC nodule formation (n=3). (H) Effect of SMAD2 or SMAD3 siRNA knockdown in MCs on nodule

formation (n=3) with immunoblots demonstrating degree of knockdown. [DharmaFECT (DH), non-targeting siRNA control (siCP), siSMAD2 (siS2), siSMAD3 (siS3), total SMAD2 (tSMAD2), total SMAD3 (tSMAD3)]. Error bars represent 1 s.d. NS, not significant; ***, $p<0.001$; **, $p<0.01$: 2-tailed Student's t test.

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Figure 5.

(A) Alterations in TGFβ/BMP receptor expression in MC mono-3D culture, in response to TGFβ (10 ng/ml) and BMP7 (100 ng/ml) stimulation and co-stimulation, measured by quantitative reverse transcriptase PCR analysis (n=3; error bars represent 1 s.d.; ***, p<0.001; *, p<0.05: 2-tailed Student's *t* test). (B) Phospho-SMAD (1, 2 and 3) activity in glomeruli from normal human kidney, diabetic nephropathy or focal segmental glomerulosclerosis (FSGS). Representative immunohistochemistry images and quantification by percentage of positive nuclei per glomerulus (diabetic nephropathy, n=3; FSGS, n=3; control tissue, n=6). *p* values are for comparison of diseased to normal tissue, using 2-tailed Student's *t* test.

Figure 6. Glomerulosclerotic phenotype in 3D tri-culture of human GECs, MCs and podocytes is prevented by ALK5 and CTGF inhibition, but not by BMP7.

(A) TGFβ-induced nodules shown by phase microscopy (red arrows). (B) Immunofluorescence of nodule, showing loss of network formation by GECs (labelled with ULEX-FITC), detachment of podocytes (labelled with PKH-Red) with loss of association with GECs, and increased number of MCs (labelled with CAMC-Blue) after 24 hours of treatment with TGFβ. (C) Immunofluorescence image of non-nodular area after 24 hours' TGFβ treatment, showing GEC (labelled with ULEX-FITC) network loss, and loss of podocyte association with GECs (podocytes labelled with PKH-Red, white arrows). MCs (labelled with CAMC-Blue) are distributed throughout the field. (D) Effect of BMP7 on TGFβ-induced nodule formation in tri-culture (n=3). (E) Effect of ALK5 inhibition & CTGF neutralising antibody (nAb) on TGFβ-induced nodule formation and collagen type I alpha1 (COL1a1) and type IV alpha1 (COL4a1) RNA quantification in tri-culture (n=3). Error bars represent 1 s.d. NS, not significant; ***, p<0.001; *, p<0.05: 2-tailed Student's *t* test.