MODELLING HUMAN GLOMERULOSCLEROSIS *IN VITRO* USING A 3-DIMENSIONAL TRI-CULTURE SYSTEM

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Category: Renal pathology. Experimental and clinical.

Introduction:

Renal fibrosis, a hallmark of chronic kidney disease (CKD) regardless of disease aetiology, is characterised by excessive extracellular matrix (ECM) deposition, abolishing fine structure and impairing function. Scarring occurs within the tubular interstitium and glomeruli; glomerulosclerosis is a feature of diabetic nephropathy (DN) and idiopathic focal segmental glomerulosclerosis (FSGS), and many other primary renal and systemic kidney diseases.

Glomerulosclerosis involves three glomerular cell types; the role of the mesangial cell (MC) is often termed the "cornerstone" of the disease because the enlarged mesangial compartment is a major hallmark of glomerulosclerosis. This enlargement is due to a combination of MC matrix deposition, hypertrophy and proliferation. Glomerular endothelial cell (GEC) apoptosis is related to the loss of glomerular capillaries, and thus loss of glomerular function. Loss of the podocyte cell type is a major problem because this highly differentiated cell type may have limited ability to proliferate in situ. Podocyte changes provide an early indication of glomerular disease and have a key role in glomerulosclerosis.

The context of a cell is important in its behaviour, and by culturing cells in 3-dimensions (3D) the environment is much more akin to in vivo. 3D co-culture creates a more relevant system than 2D culture, and allows examination of crosstalk between cell types that is vital for many cellular processes. 3D culture using human renal cells enables the modelling of disease to explore disease mechanisms and test therapeutic targets in a relevant human system, avoiding the need for animal models.

We describe the first 3D system where human GECs, MCs and podocytes are cultured together in a collagen I and fibronectin matrix. When the three glomerular cells types are seeded at a specific ratio, GECs form vascular-like networks with which MCs and podocytes interact. Cytokine treatments, such as transforming growth factor-β (TGF-β), induce nodule formation, which simulates fibrosis, resulting in a human *in vitro* system that can be used to study the pathogenesis of glomerulosclerosis.

Objectives:

This project tests the hypothesis that 3D culture of three glomerular cell types can be used to model glomerulosclerosis, better understand disease pathogenesis involving cross-talk between the glomerular cell types, and identify targets to prevent/reverse fibrosis.

Methods:

3D tri-culture of human glomerular cells was achieved by suspending cells at a specific ratio within a matrix consisting of rat collagen I and human plasma fibronectin. After a brief incubation at 37ºC the gel matrix polymerised and a tri-culture media formulation was added, which would contain any treatment. Incubation at 37ºC allowed cells to form glomerular like structures (Figure 1). RNA was extracted from 3D tri-cultures for real time quantitative PCR (RT qPCR) experiments to analyse gene expression. Small interfering RNA for receptor mediated SMAD, SMAD2 and SMAD3, were used to knock-down this TGF-β signal mediator in MCs. Confocal microscopy as well as scanning electron microscopy (SEM) were employed to examine the structures formed by 3D tri-cultures.

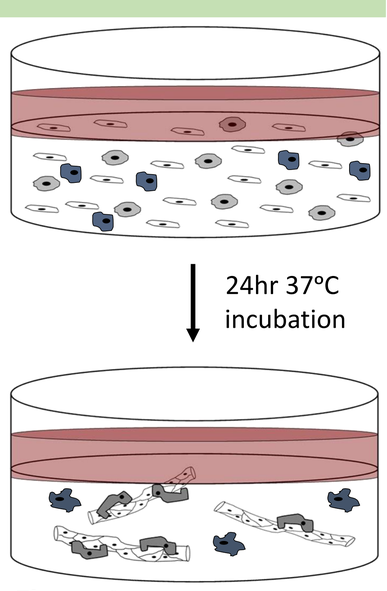


Figure 1. Rudimentary diagram depicting the 3D tri-culture of human glomerular cells.

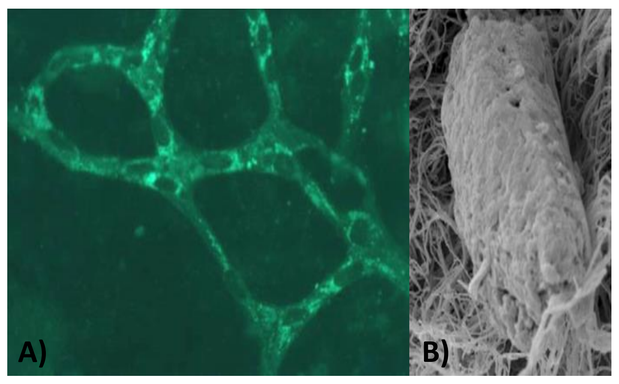


Figure 2. (A) GECs in 3D monoculture formed a vascular network, cells were stained using fluorescein-labelled *Ulex europaeus* agglutinin I (ULEX), X40 magnification. (B) SEM of GEC network in 3D monoculture where lumen and fenestrations are visible.

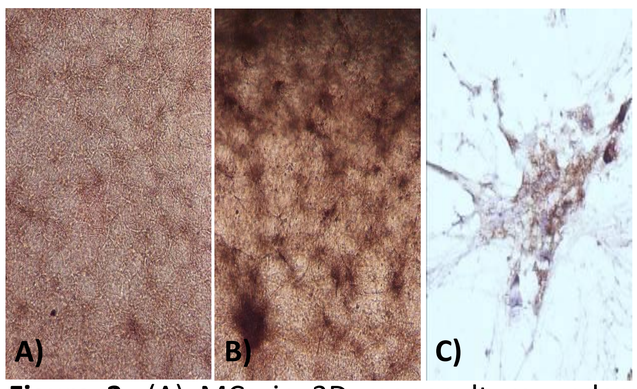


Figure 3. (A) MCs in 3D monoculture under control conditions, B) when treated with TGF-β and nodules are formed, X10 magnification. (C) Nodule with human collagen IV staining of a 3D MC monoculture section.

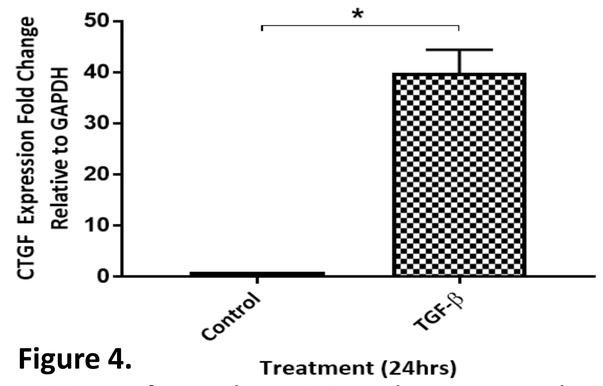
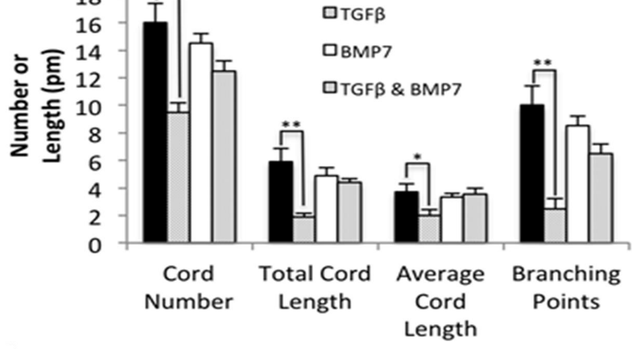
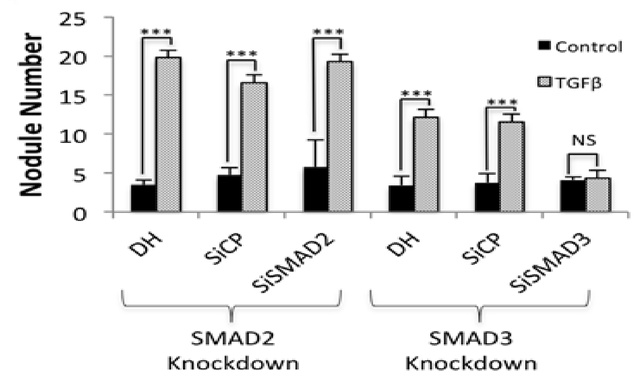


Figure 4. qPCR performed on 3D podocyte samples, ΔΔCT analysis to look at the relative fold-change of CTGF against housekeeping gene GAPDH with TGF-β treatment N=3, SEM error bars, Paired t-test p-value 0.0132.





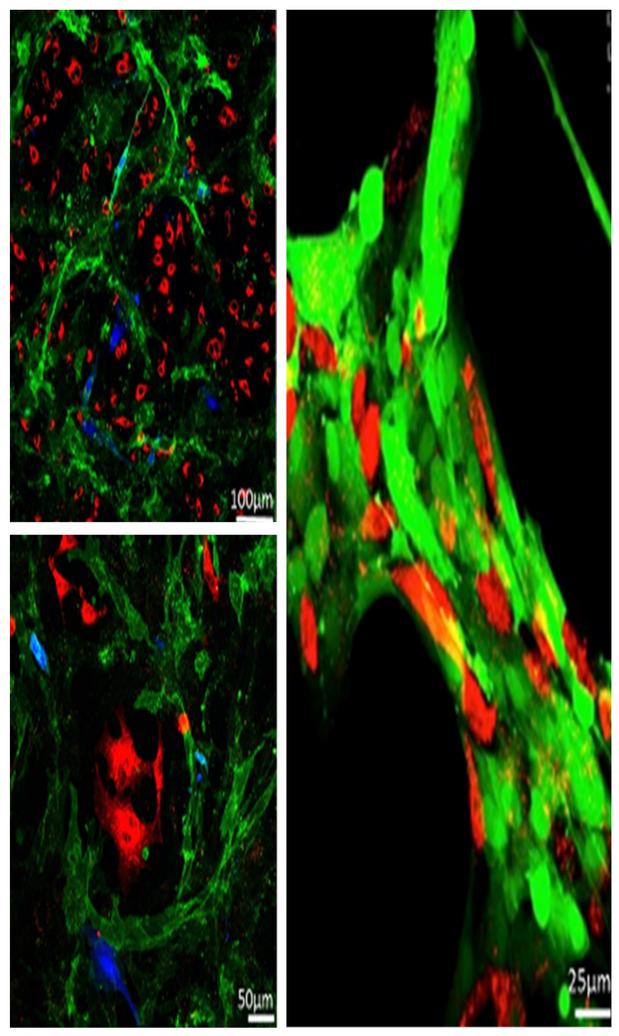


Figure 6. Images of fluorescently labelled 3D tri-cultures. GECs (green), podocytes (red), and MCs (blue). (A =10x magnification, B and C = 20x magnification)

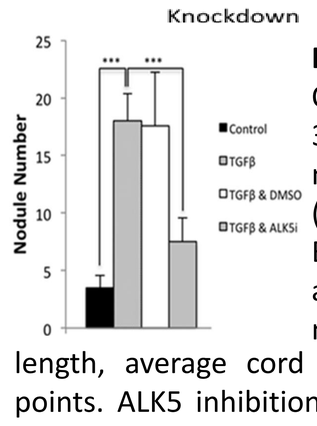


Figure 5. Quantification of GEC 3D monoculture network arborisation (A) with TGF-β and/ or BMP7 treatments, assessed by cord number, total cord length, average cord length and branching points. ALK5 inhibition (B) on 3D MC monoculture on nodule formation. SMAD2 or SMAD3 knock-down (C) effect upon 3D monoculture MC nodule formation. All data at n=3 with SD error bars and two-tailed Student's t-tests.

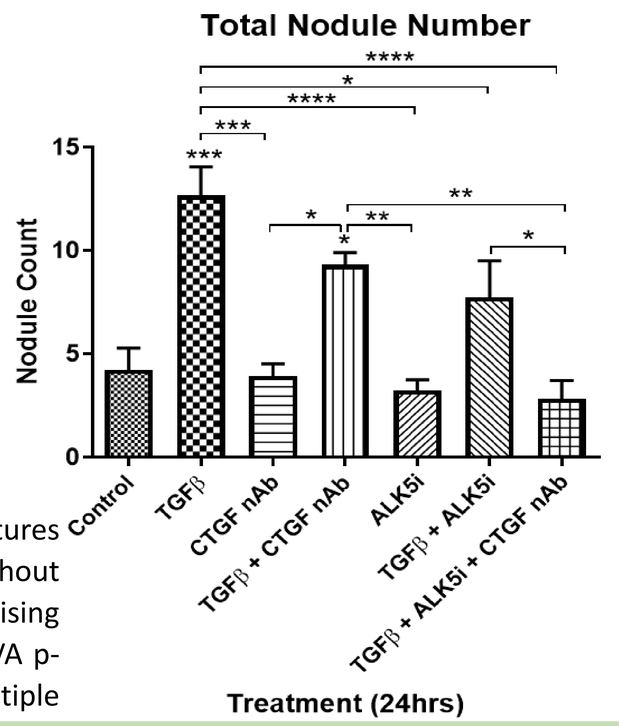


Figure 7. Nodule formation in 3D tri-cultures treated with TGF-β (10ng/ml), with or without treatment with ALK5i and CTGF neutralising antibody. N=4, SEM error bars, One-way ANOVA p-value <0.0001 (****), followed by Tukey's multiple

Results:

In 3D monoculture each cell type alters its phenotype in response to TGF-β, which has been implicated as a mediator of glomerulosclerosis. GECs form a lumenised vascular network (Figure 2), which regresses in response to TGF-β. MCs respond to TGF-β by forming glomerulosclerotic-like nodules with matrix deposition (Figure 3). TGF-β treated podocytes do not change in morphology, but demonstrate increased connective tissue growth factor (CTGF) gene expression (Figure 4). BMP7 antagonizes TGF-β signalling, and is as a possible target in kidney disease. In 3D monoculture TGF-β mediated network regression was prevented by BMP7, whereas TGF-β induced MC nodule formation is prevented by SMAD3 siRNA or ALK5 inhibition of the type I TGF-β receptor, but not by SMAD2 siRNA or BMP7 (Figure 5). In 3D tri-culture GECs, podocytes and MCs form a vascular network in which GECs and podocytes interact intimately within a matrix containing MCs (Figure 6). TGF-β induces nodule formation, but combined inhibition of ALK5 and CTGF is required to prevent TGF-β induced nodule formation in tri-cellular cultures (Figure 7). The system has been used to study the effect of other cytokines on TGF-β. For example, in 3D tri-culture TNF-α and TGF-β synergise to increase IL-8 and MCP-1 production, whereas TNF-α prevents TGF-β induction of COL1A1 (data not shown).

Conclusions:

Glomerulosclerosis and renal fibrosis involve multiple cellular events in their pathogenesis, with complex pathways and mechanisms at play. Renal fibrosis cannot be established by a distinct cell type alone.

In 3D tri-culture glomerular cells require both inhibition of ALK5 and CTGF to prevent nodule formation, whereas ALK5 inhibition inhibits nodule formation in 3D MC monoculture. Podocyte CTGF gene expression is upregulated by TGF-β, indicating they are a source of CTGF and contribute to the fibrotic response. The role of SMAD3 in the TGF-β signalling pathway is supported by siRNA and patient data, while BMP7 does not prevent fibrosis in the 3D tri-culture.

This data supports the use of this system of 3D tri-culture human glomerular cells in the study of glomerulosclerosis; providing better translatability to the human kidney.

References: Böttinger, E.P. and M. Bitzer, *TGF-ß Signaling in Renal Disease.* Journal of the American Society of Nephrology, 2002. **13**(10): p. 2600-2610.
Waters, J.P., et al., *In vitro self-assembly of human pericyte-supported endothelial microvessels in three-dimensional coculture: a simple model for interrogating endothelial-pericyte interactions.* J Vasc Res, 2013. **50**(4): p. 324-31.

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Renal pathology
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DOI: 10.3252/pso.eu.54ERA.2017
ePosters supported by F. Hoffmann- La Roche Ltd.
54th ERA-EDTA CONGRESS Madrid, Spain June 3rd-6th 2017
PosterSessionOnline.com