Mechano-chemical Insights in Diabetic Kidney Disease through 3D Pathotypic Model of Mesangium

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ABSTRACT

The mesangium is a crucial microenvironment in the kidney. It consists of mesangial cells and extracellular matrix that lends structural integrity to the glomerulus and aids renal filtration. The mesangial cells function in a delicate balance of matrix mechanics and chemical cues to engage in matrix formation, cell interactions, and cytokine production. Irregularities such as diabetes disturb this delicate balance leading to declining kidney function and kidney failure. While chemical and molecular studies on mesangium during diabetic kidney disease (DKD) are abundant, little is known about how the changing matrix mechanics affect the mesangial function. Here we demonstrate the co-stimulatory effects of chemical cues and matrix properties within the mesangial niche afflicted with DKD. To avail control of both mechanical and chemical parameters typical of DKD, we used photo-cured gelatin methacryloyl hydrogels to emulate mesangium in different disease stages. We simulated soft and stiff matrices to mechanically match mesangium in healthy and long-term DKD with fibrosis conditions. The mechanical properties play a dominant role over chemical factors in $\alpha-$smooth muscle actin formation. This coincided with a reduction in mesangial cell processes and motility, crucial for cell interactions. The fibrotic matrix also profoundly influences collagen IV expression, potentially resulting in a thickened renal basement membrane around capillaries, reducing renal filtration efficiency. The study implies that the mechano-chemical dual input in late-stage DKD causes an accelerated decline in glomerular function. The finding consolidates viable reasoning for therapeutic challenges in late-stage kidney disease and directs future studies to find the missing pieces in understanding kidney disease through such in-vitro pathotypic models.

INTRODUCTION

One in 10 adults today have diabetes globally and one third of them are estimated to develop progressive diabetic kidney disease (DKD)[1]. Current therapy for DKD can only slow down the kidney damage but cannot fully treat or reverse it especially when the disease has progressed. Thus, understanding the right therapeutic tools to target the irreversible factor of the disease is needed. The answer lies in understanding the cause of the complexity prevalent in the late-stage disease afflicted tissue. Chemical factors like high glucose condition and subsequent cytokine production initiates and enables progression of the disease. Since there are other players in the pathological machinery as the disease progresses, straightforward approaches targeting the reduction of sugars and inflammatory cytokines is not enough to fully revert the damage. Untangling these complex manifestation of the disease is important and this invites understanding the role of mechanical cues that can affect the kidney function. The chemical cues in DKD triggers fibrogenic response that leads to extracellular matrix (ECM) accumulation by ECM producing cells like the mesangial cells. ECM homeostasis is achieved by the fine balance between matrix production and matrix digestion in healthy physiology. However in DKD, the elevated metabolism and immunogenic signals, tips this balance towards increasing ECM accumulation leading to fibrosis. Accumulation of fibrous ECM components like Collagen-I increases the stiffness and density of the matrix. Collagen-IV, on the other hand, contributes to increasing thickness of basement membranes surrounding glomerular capillaries. Thus, given this plurality of mechanisms, establishing clear cause-effect relationships between mechano-chemical cues and pathological responses can go a long way towards effective therapy for complete restoration of kidney function. Currently used biological models do not provide the opportunity to study such mechano-chemical environments resembling definite disease stages of DKD.

Today cause-effect relationships studies of various factors leading to DKD are based on 2D cell culture, animal models and clinical samples. 2D culture provides good control but cannot emulate 3D tissue environment. Animal models provide platform for complex studies, but can be too challenging to establish direct causality. Clinical samples do not allow study of such long-term chronic diseases. 3D cell cultures that simulate organ-like conditions bridge the gap by providing a balance between complexity and real-time investigation with good controls to establish clear cause-effect relationships. Kidney mesangial cells are a crucial component of the kidney filtration unit i.e., glomerulus and operates at the delicate intersection of mechanical and chemical players. A key feature of DKD is mesangial expansion which includes proliferation of mesangial cells and the

extracellular matrix. Mesangial cells produce the glomerular matrix comprising type IV collagen, fibronectin, laminins, and proteoglycans[2–4]. Mesangial cells thereby helps provide structural support to the glomerular capillary loop[5–7]. They play important roles in inflammatory responses to injury and stress. Specialised functions of mesangial cells include phagocytotic activity[8], clearing proteins lodged in the glomerulus, and facilitating the turnover of the glomerular basement membrane. They play surveillance roles by reacting to innate immune stimuli to injury by modulating ECM components and producing cytokines to direct immune recruitment. Additionally mesangial cells have internal mechanism of producing angiotensin in high glucose conditions, which in turn regulates matrix production[9]. High glucose also induces mesangial expansion and fibrotic manifestation[10] leading to scarring. scRNA-Seq study found that different types of injuries can induce different combinations of matrix and chemokine gene expression by mesangial cells[11]. Mesangial cells interact with all other key players of glomerular filtration like endothelial cells, epithelial cells, and basement membrane. Further, the mesangial expansion leading to fibrosis via TGF$-\beta$ pathway[7] leads to reduction of glomerular filtration rate. Thus, understanding the mechanisms of this mechano-chemical cross-talk and its consequences could lead to a better understanding of glomerular disease and provide a route to the development of treatments that specifically target mesangial cells.

Here we study the differential role of mechano-chemical cues, in the early and late stages of DKD using 3D cell culture of mesangial cells in disease-like or pathotypic conditions (Figure 1 (A-B)). This can contribute towards resolution of long-term chronic kidney diseases. Additionally, unravelling the control of mesangial cell response can become a driver of chronic kidney disease and fibrosis. Currently, however, tools to monitor and detect mesangial cell activity and to manipulate mesangial cell function are lacking[12]. Considering the pivotal role of the mesangium, such models tailored for imaging, monitoring, and manipulation of this intricate structure holds the potential to significantly advance research in the field of kidney diseases.

RESULTS

The study focuses on evaluating the effect on the mesangial cells on hydrogel matrix with high glucose and TGF$-\beta$ to chemically emulate DKD. 5% and 10% GelMA hydrogels with stiffness of (2.5-3 kPa) and (30-35 kPa) respectively (done previously[13]) are used to elastically emulate healthy and fibrotic mesangium. As the glomerular stiffness is about 3kPa[14], 5% GelMA matrix is designated here as the healthy matrix. As fibrosis associated with diabetes increases the stiffness of the matrix[15], we considered 10% GelMA hydrogels as the fibrotic matrix.

Influence on cell-matrix interaction

We studied the mesangial cell activity in a diabetic background with high glucose conditions in four cases, (1) healthy mesangial matrix (soft 5% GelMA), (2) healthy mesangial matrix with TGF$-\beta$, (3) fibrotic mesangium (stiff 10% GelMA), (4) fibrogeneic mesangium with TGF$-\beta$. The presence of high glucose is already a known trigger for mesangial expansion and fibrogenic manifestation[10]. Here we determined, given the diabetic background, how the matrix stiffness and present of the master fibrotic cytokine TGF$-\beta$ affects the mesangial cell morphology and functions. We determined that both TGF$-\beta$ and stiffening matrix contribute to reducing the cell matrix interaction (Figure 1 (C-F)) and the insets in Figure 1 (G-J). We observed that both TGF$-\beta$ and stiff matrix individually contributed in reducing the cell-matrix interaction, but stiffening of the matrix had a more dramatic effect resulting in cells clustering with one another. We quantified the cell matrix interaction by using percentage of compactness (tight clusters have highest values) as the measure (Figure 1 K). We found that healthy matrix had the lowest compactness and the fibrotic matrix with TGF$-\beta$ treatment had the highest compactness.

Influence on mesangial processes

The length of the processes of the mesangial cells can indicate their ability to interact with other glomerular components and we found that high fibrotic matrix reduces the length and branching of these processes (Figure 1 L). Treatment of TGF$-\beta$ reduced the length of these process ever so slightly but not so significantly (Figure 1 L). Similarly, the length of the processes in the fibrotic matrix were similar irrespective of treatment with TGF$-\beta$, but were significantly different from that of the healthy matrix. Also of interest we found that on 2D glass surfaces prominent process usually seen in mesangial cells in in vivo were missing and only sheet-like lamellae was seen (SI Figure 1).

Influence on cellular motility

Based on the above observations, we conclude that the matrix stiffness has more dominant and dramatic effect on mesangial cell behaviour, morphology, and matrix interaction. Thus we evaluated the effect of the matrix stiffness on cell motility which is an important property of mesangial cell function in maintaining glomerular function. We observed that the mesangial cells on the healthy matrix displays direction plasticity which implies that cells multiple branched heads and directional flexibility to move in the matrix in a scavenging-like mode (Figure 2 (A)). On the other hand, in the fibrotic matrix, the mesangial processes displayed unidirectional motility where the cells were only moving collectively in a single direction following the leader cell. Further to quantify the directional plasticity we measure the angular protrusions of the cell processes during the motility in a

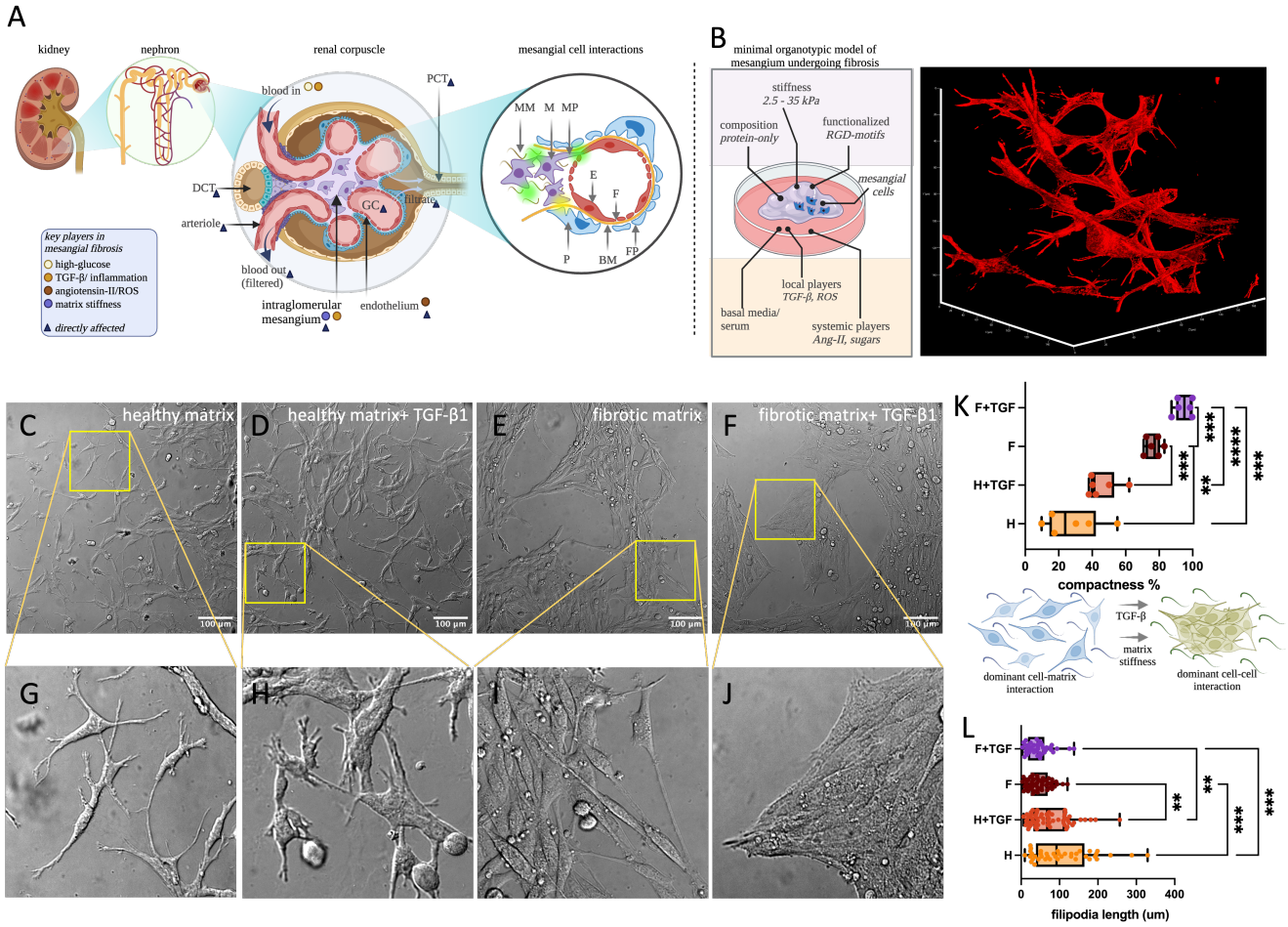

Figure 1. Mesangial cell-matrix interaction varies with mechano-chemical stimuli.
(A) The mesangium is integral to the development and sustenance of the glomerular capillary structure and function. Mesangial cells interact with different cells, matrices, and chemicals to maintain healthy kidney function and also respond to injuries and stresses. MM-mesangial matrix, M-mesangial cells, MP-mesangial processes, P-podocytes, FP-foot processes, E-endothelial cells, F-fenestrations.
(B) (Left) This delicate and complex mesangial environment is simplified using 3D culture. (Right) Actin labeled 3D reconstruction of mesangial cells in the 3D hydrogel environement.
(C,E) The distribution of cells in the matrix in the healthy and fibrotic matrix, without inflammatory cytokine TGF$-\beta$.
(D,F) The distribution of cells in the matrix in the healthy and fibrotic matrix, with inflammatory cytokine TGF$-\beta$.
(G-J) The corresponding insets show a closer view of the cell shape and matrix interaction.
(K,L) shows how the compactness and length of processes change in different mechano-chemical conditions. Statistical test done with 2-way ANOVA, multiple comparison between each category is done. Comparisons not shown are non-significant. ns (not significant): 0.123, *: p 0.032, **: 0021, ***: 0.0002, ****: <0.0001

time frame of 24 minutes (Figure 2 (B)). We found that in the healthy matrix the directional plasticity is quite high, whereas in the fibrotic matrix the cells mainly follow a uniform path.

Influence on cell contractility

As the cell motility was largely reduced in fibrotic matrix, we studied a potential causing factor of reduced motility i.e., cell contractility. Since $\alpha-$SMA is strongly correlated to cellular contractility[16], we use its expression as a measure of contractility here. In glass substrate, we found very small levels of $\alpha-$SMA expression compared to matrix. We effect of glucose and TGF$-\beta$ on $\alpha-$SMA were observable but not prominent (Figure 2 (C-F,O)). We were able to identify differences in cells growing in the healthy (Figure 2 (G-J)) and fibrotic matrix (Figure 2 (K-N)). In healthy matrix, it was clear that TGF$-\beta$ and high glucose individually contributed to higher expression of $\alpha-$SMA (Figure 2 (O)). In fibrotic matrix, mesangial cells

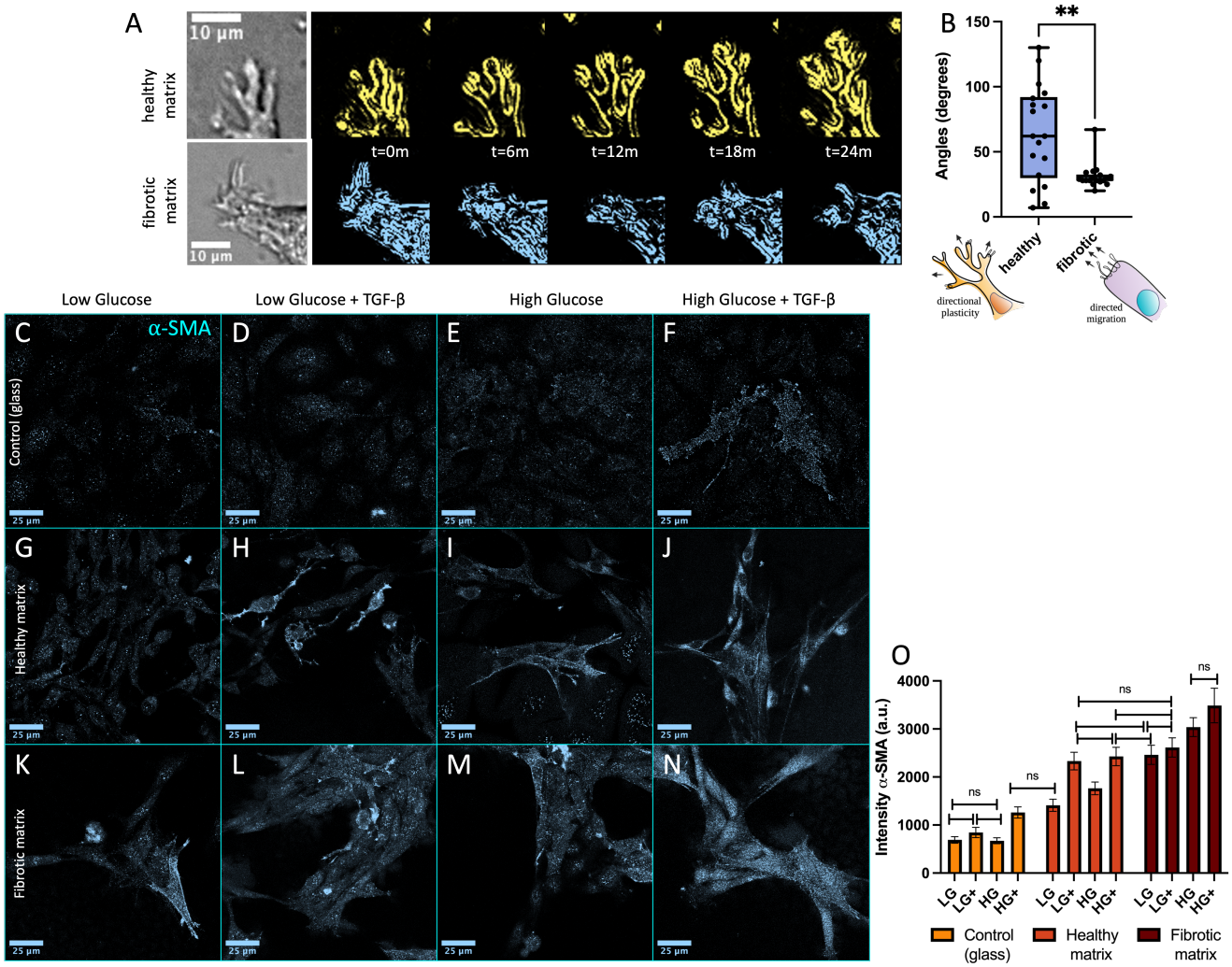

Figure 2. Changes in mesangial cell motility and contractility with mechano-chemical stimuli.
(A) Shows motility of the cells in healthy and fibrotic matrix.
(B) Higher directional plasticity in cells growing in the healthy matrix
(C-F) $\alpha-$SMA expression in glass substrate in presence and absence of high glucose and TGF$-\beta$.
(G-J) $\alpha-$SMA expression in healthy matrix in presence and absence of high glucose and TGF$-\beta$.
(K-N) $\alpha-$SMA expression in fibrotic matrix in presence and absence of high glucose and TGF$-\beta$.
(O) Plot showing that although $\alpha-$SMA expression levels quantified from confocal microscopy images. Statistical test done with 2-way ANOVA, multiple comparison between each category is done, only not-significant groups highlighted in the plot, all other groups are significantly different with p<0.0001. HG=high glucose, LG=low glucose, += added TGF$-\beta$. ns (not significant): 0.123, *: p 0.032, **: 0021, ***: 0.0002, ****: <0.0001.

showed higher $\alpha-$SMA expression than their corresponding counterparts in healthy matrix. However, the effect of effect of externally supplied TGF$-\beta$ was reduced. Another observation was the pattern of expression of $\alpha-$SMA in fibrotic matrix (SI-Figure 2). As cells tend to cluster in fibrotic matrix (Figure 1 (E,F)), we observed that in fibrotic matrix alone (without high glucose and TGF),the leader cells initiate expression of $\alpha-$SMA, while the rest of the cells express lower amounts. Further, in smaller clusters, the cluster cells have a higher $\alpha-$SMA than the cluster cells in larger clusters.

Influence on fibrogenic expression

The fibrogenic expression here is measured by the Collagen IV expression. Collagen IV is the key matrix protein that the mesangial cells deposit and contribute to the mesangium and the basement membrane. We found that in the cells grown on glass substrate, although much stiffer than our matrices, do not show clear differences due to different mechano-chemical effects

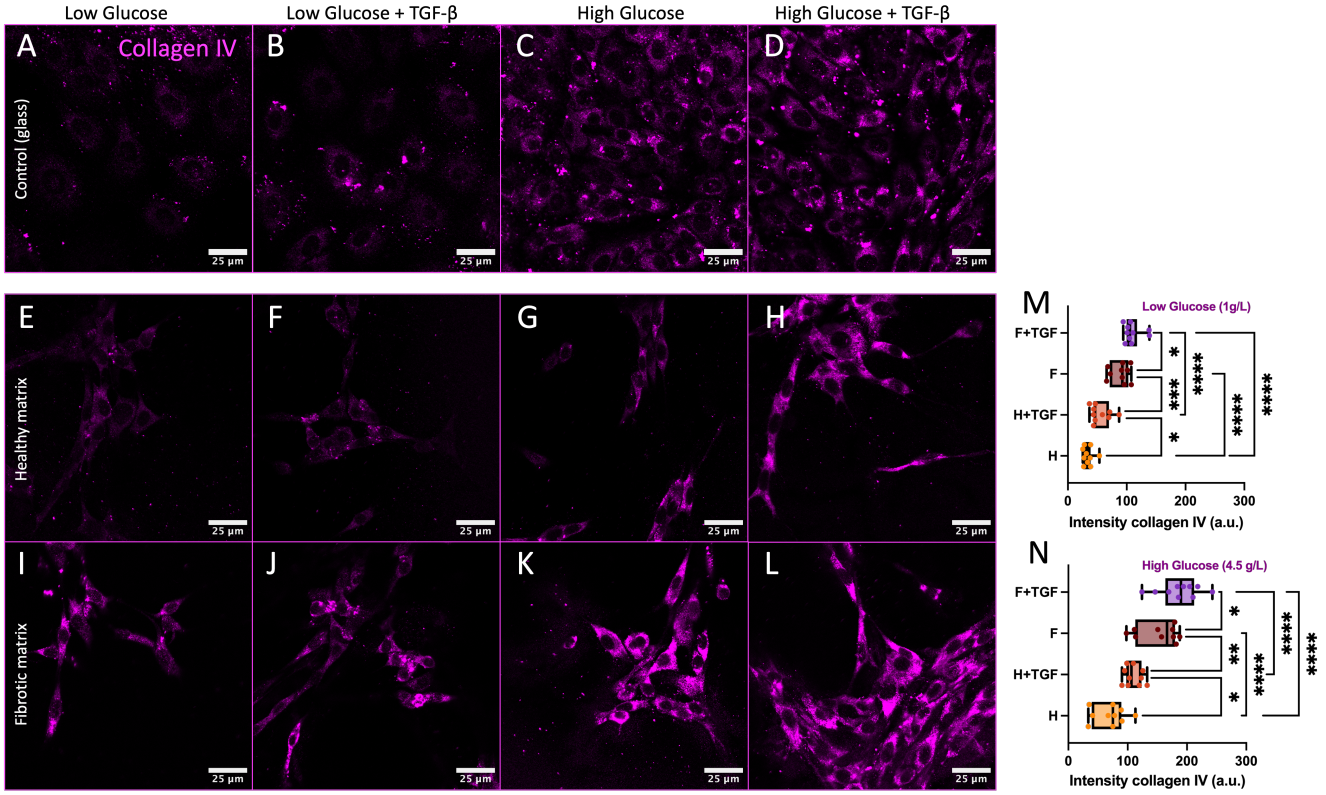

Figure 3. Effect of mechano-chemical conditions on mesangial cells towards matrix deposition
(A-D) Control Collagen IV expression in different chemical conditions when grown in glass dishes in the presence and absence of high glucose and TGF$-\beta$.
(E-H) Collagen IV expression in healthy matrix in the presence and absence of high glucose and TGF$-\beta$.
(I-L) Collagen IV in fibrotic matrix in the presence and absence of high glucose and TGF$-\beta$.
(M) Expression levels of collagen IV in low glucose conditions. H=healthy matrix, F=fibrotic matrix
(N) Expression levels of collagen IV in high glucose conditions. Statistical test done with 2-way ANOVA, multiple comparison between each category is done, only significant groups highlighted in the plot. HG=high glucose, LG=low glucose. ns (not significant): 0.123, *: p 0.032, **: 0021, ***: 0.0002, ****: <0.0001.

(Figure 3 (A-D), SI-Figure 3). Between the healthy (Figure 3 E-H))and fibrotic matrices (Figure 3 I-L)), the latter demonstrates 2x higher production of Collagen IV (Figure 3 M,N)). Glucose and TGF$-\beta$ also influence the Collagen-IV production as expected. We found that the cells in high glucose condition produced more Collagen IV than low glucose (Figure 3 (E-N), SI-Figure 4).

DISCUSSION

Recent studies on DKD have identified that it has 2 distinct phases mainly the pro-inflammatory phase initially and then, a more challenging advanced phase which is pro-fibrotic[17,18]. DKD in advanced stages manifests into interstitial fibrosis with an increased stiffness of the kidney. Thus, it is clear that the cells in the kidney are affected by both chemical and physical cues. To facilitate the study of co-stimulatory effects we studied mesangial cells, which are the ECM-producing cells of the glomerulus and have multiple roles in the proper glomerular filtration. In our study, we used two types of matrix, healthy matrix and fibrotic matrix consisting of GelMa hydrogels at two distinct mechanical stiffness emulating a healthy mesangium and a fibrotic mesangium. The healthy mesangium (lower stiffness) is used to study the early stages of DKD where the chemical cues are predominant. The fibrotic matrix (higher stiffness) resembles an advanced phase of DKD with interstitial fibrosis and increased stiffness is due to the prolonged deposition of the ECM by the mesangial cells.

In this study, we were able to validate the mesangium 3D in vitro model to express $\alpha-$SMA and collagen IV, which have been shown to behave as expected from the existing knowledge. We found that with the increase of cytokines like TGF$-\beta$ and the presence of the high glucose in the media, we found increased $\alpha-$SMA expression and stress fiber formation. This indicates

that the cell contractility increases in hyperglycemic conditions as well as in the presence of inflammation. Interestingly, for the first time, we were able to identify that increased stiffness also directly proportional to the $\alpha-$SMA and therefore, cellular contractility. TGF$-\beta$ produced in the cases of tissue injury and as well as high glucose also increases the production of ECM proteins in the mesangial cells. Our study also showed this expected outcome. Additionally, we found that there was a dramatic increase of in collagen IV production which is one of the most important proteins contributing to the glomerular basement membrane as well as dramatic increase in the mesangial matrix. It is important highlight that it is not stiffness itself that causes this dramatically increased expression of $\alpha-$SMA and collagen IV , but rather the density of the matrix protein i.e., functional stiffness, (i.e., the stiffens that can sensed and reacted to by the cells). In our case, the increased availability of RGD motifs in the fibrotic matrix which are recognized by the α_5 integrin family of cell receptors. This is proven by the fact that the fibrotic matrix with only 30 kPa of modulus has more than twice the protein expression than on a glass surface which has 1000 times higher stiffness. Another interesting observation was the collagen IV production in healthy matrix was significantly different with and without TGF$-\beta$. In a healthy matrix, TGF$-\beta$ significantly increased the production of collagen IV. However, no notable difference in collagen IV was seen in the fibrotic matrix upon treatment with TGF$-\beta$. This implies that the fibrotic matrix plays a much more dominant role in collagen IV expression. This may have therapeutic implications in that anti-inflammatory medication is not very successful during advanced stages of fibrosis. Consequently, inhibitors of α_5 integrin that will significantly hinder the cell- ECM interaction can possibly be more useful in advanced stages along with matrix-degrading enzymes. The fibrotic matrix itself can influence the mesangial cells to produce TGF$-\beta$, thereby, compounding the effect on the production of more ECM.

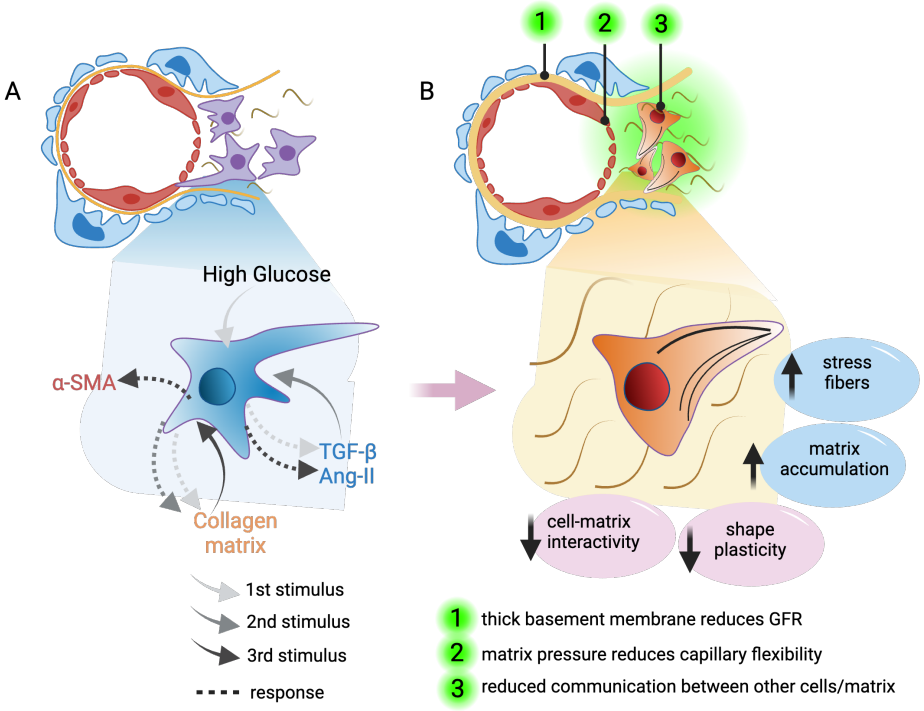

Figure 4. The potential effect of mechano-chemical co-stimulation on intra-glomerular mesangial cells to disable glomerular function in DKD
(A) The effect of high glucose initially induces enhanced production of cytokines and matrix. Continued stimulus leads to accumulation of collagen and the fibrotic matrix intesifies cytokine production.
(B) The cells in the stiff matrix with continued chemical stimulus leads to compromised cell morphology and function. This leads to increased accumulation of matrix in the basement membrane that directly reduces glomerular filtration rate.

The cellular morphology also plays a crucial role in understanding how the contractility ($\alpha-$SMA expression) affects cell interaction and motility[19]. We observed that the cells in the healthy matrix demonstrate independent cell motion as well as branched processes which are common to mesangial cells and help them interact with the other players of the glomerulus[20]. With the addition of TGF$-\beta$ and matrix stiffness, we found that the branching significantly reduced. This may have consequences in the mesangial cells interacting with the other players like the endothelial cells, basement membranes, and podocytes for its function. Conversely, it may be argued that the clustering of the mesangial cells in the fibrotic matrix may be a protective

mechanism to reduce the effect of the stiff matrix on the cells. However, this needs further investigation. Mesangial cells constantly move around in the mesangium and responding to changes to enforce the recruitment of immunological cells and produce cytokines and chemotactic factors[21]. The matrix property also defines how the degree of freedom or motility plasticity of the cells. Mesangial cells play a crucial role by actively moving in the glomerulus for performing its multitude of functions. In the healthy matrix, the cells showed high directional plasticity. In the fibrotic matrix, most of the motility tasks was burdened on the leader cells and very few independently motile cells were found. This implies that fibrotic mesangium will have a much lower availability and flexibility for crucial functions like surveillance and inter-activity with glomerulus. The cellular processes were not hampered in high glucose conditions and were minimally affected in acute treatment of TGF$-\beta$ in healthy matrix. Cellular compactness as a measure of independent cell motility was lowest in healthy matrix indicating maximum independent cell motility. The compactness was highest in fibrotic matrix with TGF$-\beta$ implying that both mechanical and chemical properties play a crucial role in regulating in mesangial cell clustering and reduce matrix interaction. The presented mesangial model for diabetic kidney disease adds value over traditional plastic and glass surfaces by providing a substrate with matrix properties matching healthy and pathological states. This provides an opportunity to study DKD which is a long-term progressive disease by isolating key phases based on matrix characteristics. Therefore, providing high degree of control and cause effect relationship, which is otherwise difficult in in-vivo models and clinical/patient samples. Live cell dynamics of mesangial cells is another opportunity to explore especially with labelfree non-linear optical imaging. The current study opens several new opportunities to understand kidney mesangium and perform more focused study to understand underlying mechanisms and identify therapeutic targets. Additionally, incorporation of other players such as endothelial cells and immune cells can be incorporated to understand their dynamics in a more holistic way.

CONCLUSION

This study emulates the kidney mesangium which is a key component of the glomerulus. We found the effects of matrix stiffness as a key factor for mesangial expansion and disease manifestation in DKD with our pathotypic system. This highlights that 2D culture studies of mesangium are inadequate to study mesangial cell function and biology. Further refinement of our model with controlled variables promises to bridge the gap between 2D cell culture and in vivo studies, enhancing our understanding of causality in kidney diseases. The findings of this study have important implications for future research on the glomerular microenvironment. By employing more cellular players and diverse molecular and spectroscopic studies, researchers can gain a better understanding of the complex mechanisms underlying DKD. Overall, this study provides valuable insights into the pathogenesis of DKD and opens up new avenues for future research in this area.

MATERIALS AND METHODS

Materials

The chemicals used in this study were rabbit polyclonal anti-α smooth muscle actin and rabbit polyclonal anti-vinculin from Abcam. Rabbit anti-mouse polyclonal anti-Collagen-IV from MD Biosciences. Goat anti-rabbit with AlexaFluor 488 and 647 from Invitrogen. Diamidino-2-phenylindole (DAPI), phalloidin - alexa 647, phosphate-buffered saline (PBS) tablets, goat serum and TGF$-\beta$ from ThermoFisher scientific. Phalloidin - Atto 565, gelatin Type A (bloom 300), methacrylate anhydride (MAA), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), sodium hydroxide (98%), hydrochloric acid (HCL), paraformaldehyde (prilled, 95%), tween-20 and commercial GelMa (80% and 40%), ninhydrin from Merck. Dulbecco's modified eagle medium (DMEM)-high glucose ($4.5g/l$), DMEM-low glucose ($1g/l$) and fetal bovine serum (FBS)-heat-inactivated, Antibiotic- Penicillin- streptomycin, ethanol and methanol from Avantor. Mouse kidney mesangial cell line DPK-KMGC-M passage 8 to 15 were used for cell culture and were commercially acquired.

GelMA Synthesis

Gelatin methacryloyl (GelMA) was synthesized in PBS pH 7.4. 10% w/v. Gelatin type A bloom 300 was dissolved in PBS and heated at 50 °C using a hotplate and stirred continuously using a magnetic stirrer. The gelatin solutions were adjusted to $pH9$ by the addition of $5N$ sodium hydroxide (NaOH) to promote the reaction of the amino groups on the gelatin. Methacrylate anhydride was added to the gelatin solutions at a feed ratio of 0.1 : 1 (ml of MAA to g of gelatin) at the rate of $0.5ml/min$ while stirring at 50 °C. The addition of MAA was divided into 6 separate times with an average of 10 min between each addition. As the reaction proceeded, the pH decreased. As such, the pH of the reaction solutions was adjusted to pH 9 after each addition for optimal reaction of gelatin with MAA. After around 1.5 h of the reaction, 1 N HCl was added to the solutions to be adjusted to the physiological pH of 7.4. All the reactions were carried out in the dark. The solutions were then filtered with a 70 mm paper filter, followed by an antibacterial filter ($0.2\mu m$). Then, the solutions were dialyzed for about 7 h against Milli-Q water for seven days at 40 °C using a dialysis membrane (12 kD molecular weight cut-off). This was done to filter out small molecules of

less than 12 kDa, unreacted MAA, salts, and methacrylic acid byproducts. After dialysis, the solutions were placed at -70 °C for 3 days through freeze-drying and finally lyophilized to obtain the dry GelMA products.

Hydrogel preparation and characterization

$50mg/ml$ LAP photoinitiator was prepared by dissolving in sterile PBS pre-warmed to 60 °C. To homogenously dissolve the LAP the mixture was pulse ultrasonicated for $5-10$ minutes and then maintained at 60 °C in a water bath. 5% and 10% (w/v) GelMA hydrogels were dissolved in sterile PBS pre-warmed to 60 °C. The mixture is made with the volume of PBS which is 90% of the total volume of the final solution. The GelMA solution was allowed to be hydrated fully in the water bath heated to 60 °C for at least 2 hours. The GelMA precursor solution was made by adding the photoinitiator to fill the remaining 10% volume of the GelMA solution such that the final LAP concentration is $5mg/ml$ (or $0.5\%w/v$). The precursor is gently mixed by pipetting such that bubbles don't form. The precursor solution is transferred to a 37 °C incubator prior to UV crosslinking such that the solution is not very viscous and spreads easily. $130\mu l$ of the precursor solution was dropped in the center of the 35mm glass bottom dish within the 20mm center well of the dish. A pipette tip was used to ensure that the solution was evenly distributed up to the walls of the well. To crosslink the gelMA, a UV LED lamp with a central wavelength of $365nm$ was used to expose the dishes containing the precursor solution for 50 seconds. 1-2 ml pre-warmed PBS at 37 °C was added to the crosslinked gelMA and kept in the incubator for $10-15$ min. The PBS is aspirated out thereafter. This step ensures all the non-crosslinked gelMA and initiator are washed out.

3D cell culture

To create the healthy and DKD mesangium in 3D hydrogel culture, 5% and 10% GelMA with an elastic modulus of $2.5kPA$ and $34kPa$ were chosen (previously done). These match the elastic modulus of the healthy and pathological conditions found in the literature. The GelMA is entirely protein-based and has RGD motifs that ensure cell adhesion like that in the tissues. Mesangial cells were cultured in low glucose and high glucose for at least 2 passages before being added to dishes with non-diabetic control and diabetic conditions. Cells are trypsinized and sufficiently neutralized with fresh media. After the GelMA is washed in warm PBS, mesangial cells are added just to the central well of the hydrogel dishes such that cells have $20-30\%$ confluence. Please add no more than $100\mu l$ in the well to avoid spilling of cells onto the other parts of the dishes. This ensures that there are no effects of the cells growing in the non-hydrogel part of the dishes. The cells are allowed to adhere to the hydrogel for 2 hours and then fresh pre-warmed low and high glucose media (1-2ml) is added to the respective dishes. The cells are allowed to spread overnight and then old media is replaced to ensure any remaining uncrosslinked gelMA/initiator is washed out to interfere with the study. The total study duration was 5 days (120 hours). TGF-$\beta 1$ is added in the concentration of $20ng/ml$ to the dishes with inflammatory-type conditions on the first day. For the next 4 days, the cells are regularly supplemented with $10ng/ml$ of TGF-$\beta 1$ to simulate a consistent level. On the 5_th day, live imaging of the cells is done to check the effect on mesangial cell processes and their cell-cell and cell-matrix interactions.

Cell Fixation and Immunolabeling

Cells in the hydrogel matrix are fixed in chilled chilled methanol. 100% methanol was allowed to be chilled in a -20 °C freezer for 30 minutes. The media was aspirated out of the dishes and washed 2-3 times in PBS. After aspirating out all the PBS, 1ml of chilled methanol was added to each dish ensuring that it covers the entire hydrogel. The dishes immersed in methanol were allowed to be fixed for 10 minutes in the -20 °C freezer. The cells were then washed and stored in a 4°C fridge with PBS. For immunolabeling, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. The detergent was washed 3 times with PBS. We ensured that the washes were done gently so that the pipette tip of the jet of reagents did not injure the hydrogel and cause a rupture. 5% goat serum in PBS was added for blocking and was allowed to sit with the cells for 30-45 minutes at room temperature. The primary and secondary antibodies were diluted as per the manufacturers' recommendation in 5% goat serum in PBS. The primary antibody was added to the hydrogel wells for 2 hours at room temperature in the dark followed by 3x PBS wash. The secondary antibody was then added for 1 hour at room temperature followed by 3x PBS wash. Other primary antibodies/secondary antibody or direct labels (phalloidin actin, DAPI) was used where multiplexing was desired. The cells were finally washed in PBS and stored in a 4C fridge in a dark condition before imaging. It is recommended that fluorescence imaging is done within a week of labeling.

Quantification of degree of substitution

The degree of substitution (DoS) for our GelMA hydrogels was determined to be 95% using Ninhydrin assay[22]. The absorbance was measured using an UV-Visible spectrometer at 570 nm wavelength. We used commercial grade GelMA with 40% and 80% DoS (Sigma-Aldrich) along with blanks (no protein) and positive control (only gelatin, no substitution) to make a standard curve. The GelMA absorbance was determined by fitting the absorbance value on the standard curve and obtaining the corresponding DoS.

Imaging and image analysis

The labelfree DIC imaging was done in GE DeltaVision microscope, using the objective lens with 10x, 0.40 Numerical Aperture (NA). The confocal imaging for measuring the expression levels was performed with Zeiss LSM 800 confocal laser scanning microscope, with a 40x, 1.20 NA water immersion objective lens. The pinhole of 1 airy units, and a laser power of 0.5% was selected. For quantification of the expression levels of the proteins (α-SMA and Colalgen IV), N=5 was used (3 biological and 2 technical replicates), ROIs were taken from 5 randomly selected images from each sample. Only the cells at the surface of the hydrogel thickness=240um, was taken to keep the height uniform to normalize quantification changes due to thickness. All labeling conditions and imaging parameters were kept as close as possible for quantification. The compactness of the cells was measured in Fiji from the DIC images. ROIs of size 100×100 pixels was selected and thresholded to identify the foreground (cells from the background). All image analysis was performed in Fiji and statistical analysis was performed in GraphPad Prism.

SUPPLEMENTARY INFORMATION

The supplementary information is provided in SI file.

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AUTHOR CONTRIBUTIONS

B.G. conceptualized the study, wrote the paper, and performed the experiments. All authors reviewed and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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