Renal Fibrosis: Mechanisms and novel therapeutic strategies

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Chronic Kidney Disease

- Renal fibrosis hallmark of Chronic Kidney Disease (CKD)
- Diverse causes: Diabetes, hypertension, hyperlipidemia, obesity, chronic inflammation, chronic infection, kidney stones, kidney cysts, immune disorders, genetic disorders (ADPKD), age; recurrent Acute Kidney Injury
- Progressive damage and reduced function over time

### Stages of Chronic Kidney Disease

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR (mL/min/1.73 m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kidney damage with normal or ↑ GFR</td>
<td>≥90</td>
</tr>
<tr>
<td>2</td>
<td>Kidney damage with mild ↓ GFR</td>
<td>60–89</td>
</tr>
<tr>
<td>3</td>
<td>Moderate ↓ GFR</td>
<td>30–59</td>
</tr>
<tr>
<td>4</td>
<td>Severe ↓ GFR</td>
<td>15–29</td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure</td>
<td>&lt;15</td>
</tr>
</tbody>
</table>

#### End-stage Renal Disease

End-stage Renal Disease is the kidney function remaining at Stage 5. Chronic kidney disease is defined as either kidney damage or GFR <60 mL/min/1.73 m² for ≥3 months. Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies.

RRT: Dialysis/Transplantation
Chronic Kidney Disease

- WHO classified CKD as a major non-communicable disease, “silent epidemic”
- Affects >850 million people worldwide, one of the most common causes of death worldwide
- Globally >3.4 million people are kept alive by RRT; only half those who need it receive treatment
- Number of patients on RRT predicted to continue to increase (aging population, hypertension and diabetes)
- In the UK estimated 3 million people are at risk of moderate/severe CKD
- Economic cost: £1.45 billion a year in England
- Patient cost: reduced QoL, premature death (patients on RRT have 2.4-19x higher mortality than age-matched population)
- Those with CKD are up to 20 times more likely to die of other causes (largely cardiovascular diseases) before reaching ESRD

Challenges:
- Currently limited treatment options – novel therapies
- Predict who will develop CKD
- Predict rate of progression
**Functional unit of the kidney: nephron**

*Glomerulus*

- Afferent arteriole
- Efferent arteriole
- Mesangial cell
- Glomerular capillary
- Mesangial matrix
- Endothelial cell
- Glomerular basement membrane (GBM)
- Bowman’s capsule
- Podocyte
- Parietal epithelial cell
- Urinary (Bowman's) space
- Proximal tubular epithelial cell

*Tubule*

1. Tubular epithelial cells
2. Interstitial fibroblasts
3. Immune cells
4. Microvascular cells

**Tubulointerstitium**

- Complex tissue ~26 different cell types
- Additional heterogeneity?
Multiple aetiologies
• Common pathology

Chronic Kidney Disease and fibrosis

• Tubulointerstitial fibrosis best predicts progression to ESRD.

Glomerulosclerosis

- Monocytes/macrophages
- Endothelial injury/activation
- Mesangial activation/proliferation transformation
- ECM deposition
- Glomerulosclerosis

Tubulointerstitial fibrosis

- Albumin/proteins
- Lipids
- Glucose
- Growth factors
- Tubular injury
- Microvascular injury
- Inflammation
- Apoptosis
- Cellular dedifferentiation
- Tubular atrophy
- Interstitial fibrosis

Normal Chronic damage

© Courtesy of Prof. A Howie.
Dept Cell Path, Royal Free Hospital

Fibrosis = pathological extension of normal wound healing

- Cellular injury/damage
- Inflammation - persistent, non-resolving
- Altered expression of growth factors and cytokines
  - Increased pro-fibrotic cytokines (TGFβ)
  - Reduced anti-fibrotic factors
- Increased interstitial cell number
- Appearance of myofibroblasts (αSMA⁺)
- Tubular atrophy and loss
  - Decreased O₂ and nutrients; hypoxia -> fibrosis
- Accumulation of ECM
  - Increased production
  - Reduced turnover (MMPs/TIMPs; PAs/PAIs)
  - Altered composition (EDA fibronectin, foetal proteins); altered cell/matrix interactions
  - Post-translational modification (cross-linking collagen by TG-2)
Most common monogenic kidney disease; ~1:800 live births

Mutations in PKD1 (PC-1) (85%) or PKD2 (PC-2) (15%)

Affects ~12 million individuals worldwide

Affects both genders, all racial, geographic and ethnic groups

50% of patients develop ESRD and require RRT (7-10% of dialysis population)
  - Wide variation in age of onset of ESRD (1-8th decade)

Characterised by extreme bilateral kidney enlargement
Cysts arise from all segments of the nephron, cyst expansion accompanied by interstitial fibrosis

Wilson P NEJM 2004
Pro-fibrotic markers in human ADPKD

E-ADPKD: Early, pre-dialysis
ES-ADPKD: End-stage
Multiple cell types activated in fibrosis

1. Tubular cells
   - Dedifferentiation
   - Proliferation
   - ECM production
   - Apoptosis
   - EMT?

2. Interstitial fibroblasts
   - Proliferation
   - Differentiation (αSMA)
   - ECM accumulation

3. Microvascular cells
   - Endothelial cells
     - Migration
     - ECM production
     - Apoptosis
     - EndoMT?
   - Pericytes
     - Differentiation
     - ECM accumulation

4. Immune/Inflammatory cells
   - Inflammation
   - Differentiation (?)

5. Progenitor cells
   - Resident
   - Circulating
ADPKD vs normal fibroblasts *in vitro*

Compared normal fibroblasts and ADPKD (PKD1 mutant) fibroblasts:

- **Altered phenotype**
  
- **Shortened cilia**

**PC-1 protein in ADPKD fibroblasts:**
- Full-length PC-1 (~460kD) undetectable
- Reduced ~250kD fragment
- Decreased expression of C-terminal ~30kD fragment
- Other fragments (~30-100kD) generally decreased with disease stage

### Table: Cilia length

<table>
<thead>
<tr>
<th>Group</th>
<th>Cilia length</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHK</td>
<td>5.8±1.5</td>
</tr>
<tr>
<td>E-ADPKD</td>
<td>4.3±0.6</td>
</tr>
<tr>
<td>ES-ADPKD</td>
<td>3.8±0.3*</td>
</tr>
</tbody>
</table>
ADPKD vs normal fibroblasts \textit{in vitro}

- Increased proliferation (basal) and differential response to growth factors

- Increased production of growth factors

- Increased GF production: CTGF, FGF, TGF\(\beta\)
**ADPKD vs normal fibroblasts *in vitro***

- **Increased collagen expression**

  ![Collagen expression graph](image)

- **Enhanced myofibroblast differentiation**

  ![Myofibroblast differentiation image](image)

- **Increased migration**

  ![Migration bar graph](image)

- **Increased collagen gel contraction**

  ![Collagen gel contraction image](image)
ADPKD vs normal fibroblasts in vitro

- Increased adhesion and spreading

- Changes in focal adhesions
ADPKD vs normal fibroblasts *in vitro*

**Dysregulated expression of ECM receptors**

**Integrins**

**Up-regulated**

- **α4**
  - NHK: 1.0
  - ADPKD: 2.0
  - *p < 0.05*

- **α5**
  - NHK: 1.0
  - ADPKD: 2.0
  - **p < 0.01**

- **β1**
  - NHK: 0.5
  - ADPKD: 2.0

- **β5**
  - NHK: 0.2
  - ADPKD: 0.8

**Down-regulated**

- **αν**
  - NHK: 1.5
  - ADPKD: 0.5

- **α1**
  - NHK: 1.5
  - ADPKD: 1.0

- **β8**
  - NHK: 1.5
  - ADPKD: 1.0

**Heterodimers:**

- ανβ3, ανβ8

**Discoidin domain receptor 2**

**Tetraspanins**

**Down-regulated:**

- TSPAN1
- TSPAN14
- TSPAN15
- TSPAN18
In vitro characterisation of ADPKD fibroblasts

- Compared to normal kidney fibroblasts ADPKD fibroblasts show:
  - Decreased PC-1
  - Cilia defects (cilia known to integrate growth factor signalling, factors relevant to fibrosis PDGF, TGFβ)
  - Stage-dependent increase in proliferation and altered response to growth factors
  - Increased myofibroblast differentiation; up-regulation of αSMA incorporated into stress fibres
  - Increased contractility
  - Increased collagen production
  - Increased matrix adhesion and spreading
  - Up-regulation of FA-associated proteins and larger FA; dysregulated ECM receptor profile

- Abnormalities reflect many of those seen in fibrotic fibroblasts from other organs
Common/unique patterns of gene expression

NHK -/+ TGFβ

- COL1A
- CTGF
- TSPAN
- HAS2
- IL11
- DDR2
- CEMIP

ADPKD

- COL
- CTGF
- TSPAN
- HAS2
- IL11
- DDR2
- CEMIP
Gene profiling of ADPKD vs normal fibroblasts

- Compare gene expression in NHK and ES-ADPKD fibroblasts
- Human Gene 1.0ST Affymetrix chip (UCL Genomics)
- Analysis by Integromics' Biomarker Discovery software

### Up-regulated genes: 507

<table>
<thead>
<tr>
<th>Name</th>
<th>Fold Change</th>
</tr>
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<tbody>
<tr>
<td>Periostin - osteoblast specific factor</td>
<td>8.03468375</td>
</tr>
<tr>
<td>Matrix Gla protein</td>
<td>7.601936667</td>
</tr>
<tr>
<td>Protocadherin 18</td>
<td>7.063819167</td>
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<tr>
<td>UDP-Gal-betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2</td>
<td>6.797520833</td>
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<tr>
<td>Oxidized LDL receptor 1</td>
<td>6.539281667</td>
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<tr>
<td>Fibroblast activation protein, alpha</td>
<td>6.456475833</td>
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<tr>
<td>Serglycin</td>
<td>6.45483</td>
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<tr>
<td>Sulfatase 1</td>
<td>6.4291425</td>
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<tr>
<td>Microfibrillar-associated protein 4</td>
<td>6.2683275</td>
</tr>
<tr>
<td>Lipid phosphate phosphatase-related protein type 4</td>
<td>6.167144583</td>
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<tr>
<td>Vestigial like 3</td>
<td>6.0196925</td>
</tr>
<tr>
<td>Sodium channel, voltage-gated, type IX, alpha subunit</td>
<td>6.001779167</td>
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<tr>
<td>Discoidin domain receptor tyrosine kinase 2</td>
<td>5.9929425</td>
</tr>
<tr>
<td>Asporin</td>
<td>5.9757225</td>
</tr>
<tr>
<td>Serpin peptidase inhibitor, clade B (ovalbumin), member 2</td>
<td>5.94260425</td>
</tr>
<tr>
<td>Anoctamin</td>
<td>5.938825833</td>
</tr>
<tr>
<td>Biglycan</td>
<td>5.933563333</td>
</tr>
<tr>
<td>Collagen, type I, alpha 2</td>
<td>5.903893333</td>
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<tr>
<td>Regulator of G-protein signaling 4</td>
<td>5.896416667</td>
</tr>
<tr>
<td>micro RNA 145</td>
<td>5.860660833</td>
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### Down-regulated genes: 556 (>2 fold)

<table>
<thead>
<tr>
<th>Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix metalloproteinase 7</td>
<td>-7.8520425</td>
</tr>
<tr>
<td>Tumor necrosis factor (ligand) superfamily, member 10</td>
<td>-7.423973333</td>
</tr>
<tr>
<td>Prominin 1 (CD133)</td>
<td>-7.293641667</td>
</tr>
<tr>
<td>CD24</td>
<td>-7.194954167</td>
</tr>
<tr>
<td>C-type lectin domain family 4, member E</td>
<td>-6.83895</td>
</tr>
<tr>
<td>Mal, T-cell differentiation protein 2 (gene/pseudogene)</td>
<td>-6.771321667</td>
</tr>
<tr>
<td>Hepatitis A virus cellular receptor 1</td>
<td>-6.700473333</td>
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<tr>
<td>Ets homologous factor</td>
<td>-6.503751667</td>
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<tr>
<td>Integrin, beta 8</td>
<td>-6.381093333</td>
</tr>
<tr>
<td>Potassium inwardly-rectifying channel, subfamily 1, member 16</td>
<td>-6.20179</td>
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<tr>
<td>Secreted phosphoprotein 1</td>
<td>-6.19344</td>
</tr>
<tr>
<td>Olfactory receptor, family 12, subfamily D, member 2</td>
<td>-6.0944</td>
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<tr>
<td>Integrin, beta 6</td>
<td>-6.038710833</td>
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<tr>
<td>Solute carrier family 17 (sodium phosphate), member 1</td>
<td>-6.0147</td>
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<tr>
<td>Epithelial cell adhesion molecule</td>
<td>-5.9800175</td>
</tr>
<tr>
<td>FAM134B</td>
<td>-5.972165833</td>
</tr>
<tr>
<td>Olfactory receptor, family 12, subfamily D, member 2</td>
<td>-5.9550325</td>
</tr>
<tr>
<td>Doublecortin domain containing 2</td>
<td>-5.861695</td>
</tr>
</tbody>
</table>

### Gene ontology (GO) enrichment analysis

- PDGFRs most common genes regulated in the array (65 relevant GO annotated biological processes, up-regulated PDGFRα and PDGFRβ feature in 11)
PDGF/PDGFR receptors in ADPKD fibrosis

- PDGFRα and β tyrosine kinase receptors interact with ligands (A, B, C, D)
- PDGF/PDGFR widely implicated in fibrosis;
- Up-regulated in a number of renal diseases
- Responses to PDGF co-ordinated by primary cilium
- ADPKD fibroblasts in vitro hyper-proliferative to PDGF
- PDGFRs elevated in ADPKD fibroblasts in vitro and in vivo
- Inhibition of PDGFR/signaling (imatinib, siRNA) attenuates fibrotic characteristics of ADPKD fibroblasts in vitro

PDGFR pathway target to slow progression of ADPKD?
- In vivo studies: Pharmacologic inhibition
  
  Fibroblast-specific deletion
  
  *Inducible Coll1a2 Cre x PDGFR floxed mice x Pkd1

Re-purposing of PDGFR TKIs in clinical use for ADPKD?
Biomarkers of renal fibrosis

- Challenges in fibrosis: to identify at-risk individuals and to predict rate of progression
- Biomarkers are under intense investigation
- Advantage of the kidney is the availability of urine as a non-invasive source of biomarkers (urinary RNAs, miRNAs, proteins, microvesicles)

**Exosomes as source of biomarkers in ADPKD**

Exosomes (30-120nm)

- **Exosomes**
  - 30-120nm vesicles
  - Originating from multivesicular bodies
  - Contain a subset of proteins, miRNAs and RNAs
  - Released into body fluids (urine, blood)/cell medium
  - Involved variety of cellular processes; cell-cell communication
  - Altered in disease

Adapted from Ko et al., Analyst 2016
Patient cohort

- Royal Free – Specialist PKD clinic with ~350 patients
- Range of stage of disease:

<table>
<thead>
<tr>
<th>CKD Stage</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70 (20%)</td>
</tr>
<tr>
<td>2</td>
<td>88 (25%)</td>
</tr>
<tr>
<td>3</td>
<td>140 (40%)</td>
</tr>
<tr>
<td>4</td>
<td>52 (15%)</td>
</tr>
</tbody>
</table>

- Urine and blood samples collected and stored (PKD Charity-sponsored Biobank)
  - Longitudinal sampling of patients over time (~6 years)
- Linked to detailed clinical data
Urinary exosome preparation

- Small volumes of urine
- Optimisation of exosome isolation from 5ml urine samples

**Ultracentrifugation protocol**

1. Add protease inhibitor to urine aliquot
   - Vortex.

2. Centrifuge samples at 300xg 15mins at 4°C.
   - Discard pellet

3. Centrifuge samples at 20,000xg 30mins.
   - Discard pellet

4. Filter supernatant to remove uromodulin network

5. Add protease inhibitor

6. Centrifuge at 197,000xg for 2.5hrs.

7. Add Isolation Solution to pellet and resuspend.

8. Centrifuge at 197,000xg for 2.5hrs to purify exosomes.

9. Remove supernatant without disturbing exosomes

**Exosome purification**

- Size (nm)

- 30-100nm

**Exosome size distribution**

- THP101
  - Filter
  - DTT

- Alix
  - 4°C
  - 20°C
**Urinary exosome protein profiling**

- Longitudinal urine samples from patients who had similar function (eGFR) at presentation but (based on clinical data) declined at different rates over 4 year follow-up.

![Diagram showing eGFR decline over follow-up](image)

<table>
<thead>
<tr>
<th>Follow-up (number of years)</th>
<th>eGFR</th>
<th>Clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
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<tr>
<td></td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

### Sample eGFR range

<table>
<thead>
<tr>
<th>Sample</th>
<th>eGFR range</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPGs</td>
<td>90-74</td>
</tr>
<tr>
<td>PGs</td>
<td>88-70</td>
</tr>
</tbody>
</table>

1) **eGFR >70**

2) **eGFR 69-50**

3) **eGFR <49**

<table>
<thead>
<tr>
<th>Sample</th>
<th>eGFR range</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPGs</td>
<td>63-59</td>
</tr>
<tr>
<td>PGs</td>
<td>64-55</td>
</tr>
<tr>
<td>NPGs</td>
<td>49-23</td>
</tr>
<tr>
<td>PGs</td>
<td>41-34</td>
</tr>
</tbody>
</table>

- Proteomics of exosomes isolated from presentation urine samples (KCL Proteomics)
- Compared protein profiles
- >2-fold difference cut-off:
  - 291 proteins up-regulated in **PGs** compared to **NPGs**
  - 30 proteins down-regulated in **PGs** compared with **NPGs**
Pathways altered in progressors vs non-progressors

- Pathway analysis (>2-fold upregulated) identified distinct patterns between those with rapid (PG) vs slow progression (NPG)
- Can distinguish PG and NPG at different starting eGFR (levels of renal function)

- Develop protein panel to distinguish rapid and slow progressors at presentation
- Use of urinary exosome profiles to determine response to treatment
- Potential to predict response to Tolvaptan (Otsuka)
New Treatments for renal fibrosis?

1. Develop drugs/biologics (antibodies) targeting pathways altered in renal fibrosis
   • New drug discovery
   • Repurposing (SGLT2 inhibitors for diabetes)

2. Developing and implementing strategies to enhance endogenous renal repair and promote generation of new nephrons

3. Engineer new organs for transplantation
   Supplement remaining tissue or replace damaged organ
   • Organoids
   • Re-seeded scaffolds (synthetic/natural)

- Studies in vivo and in vitro models of AKI/CKD have identified numerous factors and pathways dysregulated in renal fibrosis (TGFβ)

- Poor translation to the clinic

- Improved models?
  Human cell-based models
Native kidney

Decellularised ECM scaffold

SDS-based Decellularisation

SDS concentration

DNA content

Proteomic analysis: number of proteins (ECM)

Native kidney

Decellularised matrix

618 (63)

464 (58)

14 (5)
Reseeding scaffolds with human renal cells

- Normal human kidney ECM scaffolds seeded with human PTEC cell line (HK-2)
- Epithelial cells repopulate the human kidney ECM scaffold and line tubular lumens
- ECM scaffold suppresses cell proliferation
- Increases expression of cell type-specific differentiation markers
Summary

- Background to CKD and renal fibrosis
- Some insights into some of the mechanisms of renal fibrosis
- The value of in vitro human cell models in understanding the biology of fibrosis and identifying candidate therapeutic targets
- The potential of urinary exosomes as a source of biomarkers to predict progression and response to treatment
- Challenges in developing new therapeutic strategies for renal fibrosis
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