The Human Kidney Extracellular Matrix: Composition and Function

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A thesis submitted for the degree of Doctor of Philosophy

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Declaration of Originality

I, Qingyang Kong, confirm that the work presented in this thesis was accomplished by myself. I confirm that the information which was derived from other sources has been acknowledged in this thesis.
Abstract

The extracellular matrix (ECM) provides structural support for cells, regulates cell function and accumulates in renal ageing and in diseases characterised by fibrosis including chronic kidney disease (CKD) and understanding the normal ECM is key to understanding pathological changes. In the kidney, information on the ECM largely derives from candidate-based studies although recently, proteomic analysis has revealed the complex composition of the human glomerular (G) ECM. Despite the accumulation of tubulointerstitial (TI) ECM provides the best prognostic indicator of progression to end-stage renal disease in CKD, information on the TI ECM is lacking. The aim of this thesis was to characterise the human TI ECM and examine how this ECM regulates cell function. Proteomic analysis of the TI ECM from 6 human kidneys, identified 140 proteins of which 75 were newly identified in the TI matrix, 4 were newly detected in the human kidney and 2 detected in the kidney of any species for the first time. Comparison with the G matrix showed both common (126 proteins) and unique proteins (14 proteins only in TI, 38 only in G). Age-related analysis revealed 8 TI ECM proteins that increased with age, while 17 G ECM proteins increased and 3 decreased, with age. A decellularisation protocol was developed to generate human kidney cortex ECM scaffolds. These scaffolds retained a complex composition (478 proteins) and ultrastructure. Repopulation with human proximal tubular epithelia, interstitial fibroblasts and podocytes, showed that ECM scaffolds promote differentiation of all three cell types. This study provides the first detailed characterisation of the human kidney TI ECM, describes a novel protocol for decellularisation of cortical tissue cubes and shows these scaffolds promote differentiation of human kidney cells. These data enhance our understanding of the human kidney ECM and form the basis for a better understanding of renal fibrosis. These scaffolds also provide the potential to develop more complex multicellular tissue mimetics to investigate ECM regulation of human kidney cell function and for use as a platform for drug screening.
Impact statement

Chronic kidney disease (CKD) is increasing globally and imposes a significant burden socio-economic burden on individuals and healthcare systems. CKD is characterised by progressive accumulation of ECM (fibrosis) disrupting normal tissue structure and function ultimately leading to end-stage renal disease and the need for renal replacement therapy (dialysis or transplantation). Currently, there are no effective treatments and there is a pressing need for new therapies. To understand pathological changes in the ECM it is necessary to understand the composition and function of the normal ECM. The key pathological feature of CKD is the accumulation of ECM in the tubulointerstitium (TI) of the kidney but information of the composition of the ECM in this tissue compartment is lacking. This study provides the first detailed proteomic characterisation of the human kidney TI matrix and comparison of this ECM with that of the glomerulus showing similarities and differences between the matrix in the two tissue compartments, as well as identifying age-related changes in these matrices.

Understanding how the ECM regulates normal cell function is also key to understanding how those processes are altered in disease. Here a protocol was developed to produce acellular ECM scaffolds from human kidney tissue which retained a complex composition and ultrastructure. Seeding of these scaffolds with different human kidney cell types showed cell-type specific differences in behaviour on the scaffolds. In addition to providing insights into the cell biology of the kidney, these studies form the basis for developing human kidney cell-based tissue mimetics.

Academic impact is delivered through developing new collaborations and expanding knowledge of the composition and regulatory role of the human kidney matrix which could be used as foundation for future research projects. Proteomic analysis of the decellularised scaffolds also highlighted potential limitations of the widely used databases for defining ECM proteins. Impact has also been delivered through presentations at local, national and international conferences and a manuscript has been submitted for publication. Potential impact for patients of this work will be delivered
through a better understanding of the kidney ECM with the potential to identify novel therapeutic targets for fibrosis and through the development of human kidney tissue mimetics which could contribute to advancing the field of organ regeneration and provide a platform for testing novel therapeutic agents and attract research and development investment. One of the current barriers to effective translation of pre-clinical data to clinical treatments is the lack of good in vitro models which novel tissue mimetics will help address. Tissue mimetics may also have commercial impact if adopted by pharmaceutical companies for use in drug testing.
Acknowledgement

I must start by thanking my supervisors, Prof. Jill Norman and Dr. David Long, who have helped me enormously throughout my PhD studies. Prof. Jill Norman is a very kind person and has a profound knowledge of kidney matrix. Jill has helped me in every aspect during my PhD from research to personal life, especially some difficult times after my surgery. I am so fortunate to be under her supervision.

I am also grateful to Dr. David Long for his valuable advice and generous support for my studies. David is very passionate and experienced in the study of kidney disease, which has inspired me during my study.

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entire time of my PhD. Without her encouragement, it would be impossible for me to finish this thesis. I am also very grateful to my baby son, Fanping Kong, who was born in 2018 during my studies and provided me with a tremendous amount of happiness and joy. I would like to thank my Dad, Mum, my parents-in-law, and all my families for their real and unconditional love and support.
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<td>2D</td>
<td>2-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>3-dimensional</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
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<tr>
<td>BM</td>
<td>Basement membrane</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate</td>
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<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CKO</td>
<td>Conditional knock out</td>
</tr>
<tr>
<td>COL</td>
<td>Collagen</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<tr>
<td>DBA</td>
<td><em>Dolichos biflorus</em> agglutinin</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>DDR</td>
<td>Discoidin domain receptor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithiothreitol</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDAT</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
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<td>EV</td>
<td>Extracellular vesicle</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>FDR</td>
<td>False discovery rate</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>Fibronectin</td>
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<td>Glomerular</td>
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<td>glycosaminoglycan</td>
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<td>GBM</td>
<td>Glomerular basement membrane</td>
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<td>GEnC</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GGT</td>
<td>Gamma-glutamyl transpeptidase</td>
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<td>GO</td>
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<td>H&amp;E</td>
<td>Haemotoxylin and eosin</td>
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<td>HA</td>
<td>Hyaluronic acid</td>
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<td>hASC</td>
<td>Human adipose-derived stem cell</td>
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<td>Hepatitis C</td>
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<td>HKMEC</td>
<td>Human kidney peritubular microvascular endothelial cell</td>
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<td>HMDB</td>
<td>Human matrisome database</td>
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<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<tr>
<td>IFL</td>
<td>Immunofluorescence</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
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<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
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<tr>
<td>KO</td>
<td>Knock out</td>
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<tr>
<td>LAM</td>
<td>Laminin</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<td>LTBP1</td>
<td>Latent-TGFβ-binding protein 1</td>
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<tr>
<td>LTL</td>
<td><em>Lotus tetragonalobus</em> lectin</td>
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<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionisation</td>
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<tr>
<td>MALDI-IMS</td>
<td>MALDI-time-of-flight imaging mass spectrometry</td>
</tr>
<tr>
<td>MCL</td>
<td>Markov Cluster Algorithm</td>
</tr>
<tr>
<td>mESC</td>
<td>Mouse embryonic stem cell</td>
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<tr>
<td>mKSC</td>
<td>Mouse kidney stem cell</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
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</tr>
<tr>
<td>PAA</td>
<td>Peracetic acid</td>
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<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid–Schiff</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PD</td>
<td>Protocol for decellularisation</td>
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<td>Platelet-derived growth factor receptor</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<td>RCTE</td>
<td>Renal cortical tubular epithelial cells</td>
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<tr>
<td>SDC</td>
<td>Sodium Deoxycholate</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>Sodium dodecyl sulfate-polyacrylamide gel</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
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<td>sGAG</td>
<td>Sulphated glycosaminoglycan</td>
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<tr>
<td>SGLT2</td>
<td>Sodium-glucose linked transporter 2</td>
</tr>
<tr>
<td>SIS</td>
<td>Small intestine submucosa</td>
</tr>
<tr>
<td>SMART</td>
<td>Simple modular architecture research tool</td>
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<tr>
<td>TBM</td>
<td>Tubular basement membrane</td>
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<td>Tris-buffered saline</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<tr>
<td>TI</td>
<td>Tubulointerstitial</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinase</td>
</tr>
<tr>
<td>Triton</td>
<td>Triton X-100</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end-labelling</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureteral obstruction</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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<td>WT-1</td>
<td>Wilms tumour-1</td>
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<td>A2M</td>
<td>Alpha-2-macroglobulin</td>
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<td>Disintegrin and metzloproteinase domain-containing protein 9</td>
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COL8A1 Collagen alpha-1(VIII) chain
CRELD1 Cysteine-rich with epidermal growth factor-like domain protein 1
CSPG4 Chondroitin sulfate proteoglycan 4
CTSA Carboxypeptidase
CTSB Cathepsin B
CTSC Dipeptidyl peptidase 1
CTSD Cathepsin D
CTSG Cathepsin G
CTSH Pro-cathepsin H
CTSS Cathepsin S
CTSZ Cathepsin Z
CXCL14 C-X-C motif chemokine 14
DCD Dermcidin
DCN Decorin
DEFA1 Neutrophil defensin 1
DPT Dermatopontin
DSG1 Desmoglein 1
DSP Desmoplakin
ECM1 Extracellular matrix protein 1
EFEMP1 Epidermal growth factor-containing fibulin-like extracellular matrix protein 1
ELANE Neutrophil elastase
ELN Elastin
EMCN Endomucin
EMILIN1 Elastin Microfibril Interfacer 1
F3 Tissue factor
F9 Coagulation factor IX
FBLN1 Fibulin-1
FBLN5 Fibulin-5
FBN1 Fibrilllin-1
FGA Fibrinogen alpha chain
FGB Fibrinogen beta chain
FGF2 Fibroblast growth factor
FGG Fibrinogen gamma chain
FGL1 Fibrinogen-like protein 1
FLG2 Filaggrin-2
FN1 Fibronectin
FRAS1 Fraser extracellular matrix complex subunit 1
FREM1 Fraser extracellular matrix complex subunit 1-related extracellular matrix protein 1
FREM2 Fraser extracellular matrix complex subunit 1-related extracellular matrix protein 2
GANAB Neutral alpha-glucosidase AB
HPX Hemopexin
HRG Histidine-rich glycoprotein
HRNR Hornerin
HSP90B1 Endoplasmin
HSPG2 Basement membrane-specific heparan sulfate proteoglycan core protein
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Chapter 1

Introduction
1.1 Extracellular matrix

1.1.1 What is the extracellular matrix?

In general, tissues are defined as the combination of cells and their surrounding extracellular matrices (ECM) (1). The ECM is a collection of molecules produced by cells to provide three-dimensional (3D) biochemical and structural support and to determine the biomechanical properties and boundaries of tissues (2, 3). For a long time, the ECM was considered to be a static support for cells. However, studies showed that the ECM contains a variety of large structural molecules and regulatory factors, and interacts with cells embedded in it (4-6). Disruption of the ECM during development and in healthy organs leads to developmental abnormalities and pathological dysregulation of the matrix (5, 6).

The ECM can be divided into two major types: basement membrane (BM) and interstitial (stromal) matrix (5). BMs are specialised ECM structures, predominantly formed by collagen IV, laminins (LAMs), and proteoglycans (7) which provide a physical barrier separating epithelial, endothelial and mesothelial cells from the underlying connective tissue. BMs also promote cell polarity (8, 9) while still allowing diffusion and transport of gases, nutrients and signalling molecules (10). The interstitial matrix fills the intercellular spaces between mesenchymal cells and generally contains collagens (collagen I, III, V, VI, and VII), glycosaminoglycans (eg. hyaluronan) and glycoproteins (eg. fibronectin (FN), decorin) (11, 12).

1.1.2 Cell-ECM interaction

The ECM influences a wide variety of cellular processes including proliferation, differentiation, adhesion, migration and apoptosis (2). Specific receptors, including the integrins and discoidin domain receptors (DDRs), on the cell surface mediate cell-ECM interactions (12). Integrins are heterodimeric transmembrane receptors (containing an α and a β subunit) which recognise proteins in the ECM, including collagens, LAMs and FN (13). The key feature of integrins, vital for cell signalling, is the ability to transfer information from either side of the cell membrane to the other side by the connection of...
the cytosolic tails with the cytoskeleton and the interaction of extracellular domains with
the microenvironment (inside-out and outside-in signalling). For example, when the
extracellular domain is stimulated, the conformation of the receptor changes allowing the
cytosolic tail to bind the cytoskeleton and activate downstream signalling pathways (12,
14). There are important interactions between integrins and growth factor receptors, such
as epidermal growth factor (EGF) receptor (EGFR) and platelet-derived growth factor
(PDGF) receptors (PDGFR), which co-ordinate growth factor signalling and the cell-
matrix signalling (15). Different cells express different integrins, for example, in the
kidney, podocytes express integrin α3β1 and αvβ3, while mesangial cells express the
α1β1 and α2β1 integrins (14) reflecting their interaction with different ECM proteins.

Other ECM receptors, such as the DDRs, recognise and bind to specific motifs
of collagens to initiate intracellular signalling and are involved in cell polarity regulation,
cell differentiation, tissue morphogenesis and homeostasis (15). The DDR family has two
members, DDR1 and DDR2, which are the major non-integrin collagen receptors and
are transmembrane tyrosine kinase receptors (12, 16). The DDRs are widely expressed
in many organs, including kidney, during development and in adulthood (16, 17). DDR1
is predominantly observed in human epithelial cells, while DDR2 is detected in human
mesenchymal cells (16-18). Although both DDRs recognises collagen I, III and V, there
is some specificity. For example, only DDR1 binds to collagen IV and only DDR2
recognise collagen X (16, 19). Another group of matrix receptors are the syndecans
(syndecan-1, syndecan-2, syndecan-3 and syndecan-4), which are transmembrane
heparan sulfate proteoglycans (HSPG) (20, 21). Syndecan-1 and syndecan-2 are
expressed in tubular epithelial cells (22) (23). Syndecan-3 is expressed predominantly in
the nervous system (21, 24) while syndecan-4 is widely expressed in most tissues (21,
24). Syndecans interact with a wide range of components in the ECM. For example,
syndecan-1 interacts with fibrillar collagens (I, III and V) and FN (24, 25), syndecan-2
senses FN (24), syndecan-3 interacts with heparan sulfate proteins (26) and syndecan-
4 binds with tenascin-C, LAM and fibronectin (27, 28). Overall, signalling via surface
matrix receptors enables cells to sense and adapt to the changes in the ECM microenvironment and are therefore key regulators of cell function (12, 15, 21).

1.1.3 ECM in the kidney

1.1.3.1 ECM in the adult kidney

The kidney is one of the most complex organs in the body (12). The normal human kidney contains 1-2 million nephrons which are the functional units of the kidney and responsible for blood filtration, urine formation and regulation of blood pressure (29). There are three distinct histological compartments in the kidney: glomeruli, tubulointerstitium and vasculature (Figure 1.1). Each of these compartments possesses characteristic matrices (12).

![Figure 1.1 Schematic of the structure of the kidney.](http://biorender.com)
The glomerulus, responsible for filtration of the blood, contains the glomerular basement membrane (GBM) and the mesangial matrix (9, 30, 31). The GBM is made up of LAM α5β2γ1, collagen IV α3, α4 and α5 chains, entactin, nidogen, agrin and a small amount of perlecan (9). The GBM provides adhesion for the endothelial cells and podocytes (highly specialised epithelial cells which form foot processes which interact with foot processes of neighbouring cells to form the slit diaphragm (32, 33)) (Figure 1.2) (30). Byron and colleagues showed that the matrix produced by the co-culture of human endothelial cells and podocytes is more similar to human GBM than that produced by mono-cultures of each cell type in terms of both structure and composition, suggesting that endothelial cells and podocytes influence each other and both contribute to the GBM (34). This study illustrated that complex matrices are often produced by a variety of cell types. The adhesion and barrier properties of the GBM play essential roles in the filtration of urine (35). LAMs are a group of glycoproteins which consist of three chains (α chain, β chain and γ chain). LAM α5β2γ1 is required for the formation and function of GBM (30). In Lamβ2 global knock out (KO) mice, although there was some compensatory deposition of other LAMs (LAM α1β1γ1, α2β1γ1, α3β3γ2 and α5β1γ1), the mice still developed proteinuria as a result of podocyte abnormalities (36, 37). Collagen IV also
plays an important role in the GBM (12). Collagen IV has 6 different α chains (α1-6) which can form 3 different heterotrimers (α1α1α2, α3α4α5 and α5α5α6). Among them, collagen IV α3α4α5 is found in the adult GBM and forms a mesh-like network providing strength and stability (30). Col4A3 global knock out mice showed a thickened and split GBM with increased collagen IV(α1α1α2) and other LAMs (LAM α5β1γ1, α1β1γ1, α2β1γ1 and α2β2γ1) and developed proteinuria (38, 39). These studies suggested that composition and structure of the GBM are essential to maintain the glomerular filtration barrier. Other matrix proteins in the GBM include nidogens and agrin. Agrin confers a negative charge on the GBM, which prevents negatively-charged molecules, such as albumin, from passing through the filtration barrier (9). However, podocyte-specific agrin knock out (using Cre-LoxP recombination system) or treatment of wild-type (WT) mice with polyethyleneimine (to create a positive charge) showed that a significant reduction of negative charge did not cause proteinuria (40) suggesting that the role of agrin and the mechanism of albumin exclusion is much more complicated than simply charge. Nidogens, nidogen 1 and nidogen 2, are glycoproteins, however their role in the kidney is still unclear (12). Global knock out of the nidogens individually did not affect the formation of BMs (either glomerular and tubular), however, deletion of both leads to perinatal lethality (due to delayed lung development and impaired heart morphogenesis and integrity) (41-44) suggesting that although nidogens are not crucial for BM formation they have other important roles. The glomerular mesangial matrix consists of collagen IV (α1α1α2), collagen V, LAMs, FN, agrin, nidogens, perlecan and decorin (12). The LAM α1 chain is present in the adult mesangial matrix of all species (45). In Lama1 conditional knock out mice (Sox2-Cre-mediated deletion of Lama1 specifically in the epiblast lineage), there was increased proliferation of mesangial cells and expansion of mesangial matrix and mesangial cells, isolated from these mice showed increased expression of transforming growth factor (TGF)-β1 (45). These results suggest that the LAM α1 chain can regulate the function of mesangial cells and may play a role in mesangial matrix deposition via TGF-β signalling (45). Nidogens are also reported in the
mesangial matrix (46) and increased expression of both nidogens in the mesangial matrix was reported in patients with diabetic nephropathy (47) suggesting an association between nidogens and diabetes. However, the function of these proteins in the mesangial matrix is still poorly understood suggesting that the involvement of ECM in health and diseases required further investigation.

In the tubulointerstitium (TI), there are three main matrix compartments: tubular BMs (TBMs), the interstitial matrix and vascular BMs (12). In contrast to the GBM, TBMs contain LAM α1β1γ1, LAM α5β1γ1, collagen IV α1-6 chains, nidogens, perlecan, and agrin, however, it is important to note that the tubular epithelial cells in different nephron segments produce different BMs (35, 48) which may relate to their physiological functions (8, 30). Studies showed that epithelial cells are able to get signals from the underlying matrix/substrate and produce a different matrix (49, 50). For example, human renal proximal tubular epithelial cells expressed high levels of FN and collagen IV and low levels of collagen I and III when seeded onto plastic (49). Similar level of expression of collagen III and IV was detected (FN was not tested) when Madin-Darby canine kidney (MDCK) cells were seeded on collagen gel (50). The TBM establishes polarity of the epithelial cells and provides support for specialised tubular function (re-absorption and secretion of ions and macromolecules) by controlling bi-directional movement of substances through TBM between the tubule and interstitium (8, 35, 51). In Col4A1 mutant mice, the TBM appeared normal (examined by electron microscopy) suggesting a compensatory mechanism (52). While, in Col4A3 global knock out mice (mimicking Alport syndrome), enlarged and partially destroyed tubules were noted under light microscopy (38, 53, 54) suggesting that there might not be a compensatory mechanism for Col4A3 in TBM. Deletion of nidogen-1 and -2 in double knock out mice (global knock out), induced thickening or loss of TBM (in approximately 10% of double knock out mice), but had no effect on the GBM (43, 44) suggesting different roles for nidogens in the TBM and GBM. Interestingly, deletion of either nidogen individually had no effect on the TBM (41, 42). Although much interest is focussed on the GBM and understanding both the
normal and pathological GBM, these data highlight the importance of changes in TBM to kidney function. In terms of the TI matrix, published studies, using candidate-based approaches, have identified collagens I (55), III (56), IV (57) and V (58), LAMs (8, 59), FN (60), tenascin (61), TGF-β1 (62), decorin (55) and biglycan (55) as components of the TI matrix in healthy and diseased kidneys from a variety of species. There is differential expression of these proteins in different diseases, such as increased expression of collagen IV, V, LAMs, and FN in diabetic nephropathy (57, 58, 60) and more collagen 1, decorin and biglycan was observed in the biopsies of patients with interstitial fibrosis (55). Another component of TI matrix are vascular BMs, however, the composition of these BMs are not well described in the kidney (12). There is some evidence from gene mutation studies which might suggest functions of matrix proteins in the vasculature of kidney. For example, in mice with Col4A1 mutation, focal absence and fragmentation of the vascular BM in the kidney was observed and resulted in haematuria and haemorrhage, suggesting that Col4A1 is important to maintain the function of vascular BM in the mouse kidney (52). Other matrix proteins, such as LAMs, nidogens and collagen IV were also described in the BMs of different types of blood vessels (capillary, vein or artery) in different organs (63). All the data on the kidney TI ECM in humans are derived from candidate-based studies, and a comprehensive analysis of the human TI matrix is still needed.

In addition to protein components, the ECM also contains non-protein components including polysaccharides, glycosaminoglycans (GAGs), water, metal ions, lipids and nucleic acids (11, 64), with GAGs being the predominant category (65). GAGs are long linear polysaccharides consisting of repeating disaccharide units or repeating galactose units with 2-amino-2-deoxysugar (66). Among the 4 classes of GAGs, differentiated by the structure of the core disaccharide: heparin/heparan sulphate GAGs, chondroitin sulphate GAGs, keratan sulphate GAGs and hyaluronic acid (HA), HA is the only GAG which is not linked to a protein core (65). HA is a negatively-charged, linear, high molecular weight GAG and interacts with a variety of cellular receptors (eg. CD44,
CD54, RHAMM (the receptor for HA-mediated motility), and HARE (the HA receptor for endocytosis)) and proteoglycans (eg. aggrecan) to regulate cell functions, such as proliferation and cell locomotion (11, 67). In the healthy human kidney, HA is mainly found in the medulla, where it is involved the regulation of fluid homeostasis via its capacity to reabsorb water, with only low levels of expression in the cortex (67). Water is another non-protein component in the kidney ECM. Water is retained in the ECM by the interaction between water molecules and the charge on polysaccharides such as HA (68). The composition of polysaccharide-glycoproteins dictates the degree of hydration of the ECM (11, 67, 68). Metal ions are also found in the ECM and extracellular metal ions are thought to as cofactors for integrins and thereby regulate cell behaviour (69). For example, it has been shown that Ca2+ and Mg2+ ions are present in the matrix and are involved in integrin-fibronectin interactions (69, 70). It also has been shown that manganese ions induce the formation of stress fibres inhuman endothelial cells (71). These data suggest that metal ions are not only present in the matrix but also play a key role in the cell-matrix signalling and the regulation of cell behaviour. A major source of additional non-protein components in the ECM are extracellular vesicles (EVs) (72), a heterogenous family of membrane-bound vesicles released by cells. EVs contain proteins, polysaccharide, lipids and nucleic acids and function as a means of transferring information between cells (72). EVs can interact with ECM components via their surface adhesion ligands and receptors such as integrins (with laminin and fibronectin) and CD44 (with HA) and may be retained in the ECM (73-75). EVs are produced by different renal cell types and carry a variety of different cargo including RNAs (76, 77) and HA (78). EVs have been identified in the tubulointerstitial ECM of the murine kidney (76). Taken together, these data highlight the potential importance of non-protein components in fully understanding the composition and function of ECM in the kidney.

The ECM is dynamic, and its composition varies during the life of an individual (79, 80). One question is how the kidney ECM changes with age which may be relevant to understanding age-related changes in kidney function as well as pathological changes.
Some information is available from candidate-based approaches. For example, renal interstitial fibrosis, glomerulosclerosis and arteriolosclerosis are associated with ageing in marmosets (16 years compared to 3 years; maximum lifespan 20 years) with an age-related increase in collagen III (81). There is also evidence from rodents and humans, that the composition of the ECM alters as the kidney ages. Immunohistochemistry (IHC) showed increased expression of collagen IV in the glomeruli of aged mice (27 months) compared with young mice (3 months) (82). Another study showed an increase in the mesangial matrix (83) and deposition of collagen IV and heparan sulphate proteoglycans (HSPG) in Bowman’s capsule (84) in 27 months C57/Bl6 mice compared with 3 months animals. Information on age-related changes in the TI is sparse and available data are largely derived from animal models. FN, thrombospondin (85) and collagen IV expression (86) are all reported to be increased in aged rat kidneys (24 months) compared with young animals (3-6 months). In addition, Sirius red staining and immunoblotting, showed elevated collagen I and III in the TI of the kidneys of 26-32 month C57Bl/6 mice compared to 4-6 months animals (83). A 2-4-fold increase in transglutaminase 2 (TGM2) expression was detected by Western blotting and immunofluorescence (IFL) in ageing FVB mouse kidneys (22-24 months) compared to 3-6-month kidneys (87). Western blotting of proteins from human kidney cortex showed that levels of collagen I, but not collagen IV, increased significantly with age (from 19 to 92 years) (88). The available data highlight age-related changes in the ECM and suggest more comprehensive analyses are warranted.

1.1.3.2 ECM in chronic kidney disease

In a clinical setting, chronic kidney disease (CKD) is divided into five stages based on glomerular filtration rate (https://kdigo.org/). A key feature of CKD is the accumulation of ECM which disrupts normal tissue architecture leading to the progressive loss of kidney function and ultimately, organ failure (89-92). Accumulation of ECM occurs as a result of both increased production of matrix proteins and decreased degradation (93). In CKD, multiple cell types contribute to the accumulation of ECM (12,
For example, in the glomeruli, mesangial cells are activated and produce ECM which can lead to mesangial sclerosis (94). Podocytes and endothelial cells usually contribute to the thickening of GBM in fibrosis (95). In the tubulointerstitium, tubular epithelial cells contribute to the thickening of TBM, however, a major contributor is the matrix-producing myofibroblasts (95). These matrix-producing myofibroblasts are thought to originate from a variety of different cell types, including interstitial fibroblasts, tubular epithelial cells, microvascular cells and bone marrow-derived cells (96). Myofibroblasts secrete a variety of matrix components, including collagens and FN. The accumulation of ECM can also be a result of impaired ECM turnover which is regulated by a number of enzymes of which the major ones are the matrix metalloproteinases (MMPs) and the Plasminogen Activators (uPA/tPA) (12, 90, 97). The activity of these enzymes is regulated by their endogenous inhibitors, the Tissue Inhibitors of Metalloproteinases (TIMPs) and Plasminogen Activator Inhibitors (PAIs), respectively. Recent studies have shown that the roles of different TIMPs in fibrosis are complex. TIMP2 and TIMP3 knock out revealed distinct roles for these two proteins in kidney fibrosis (98). MMP2 activation, collagen I and II expression, and TGF-β were significantly increased in Timp3−/− mice (global knock out), but not in Timp2−/− mice (global knock out), subjected to unilateral ureteral obstruction (UUO), suggesting that TIMP3 might have a protective role in fibrosis whereas TIMP2 might contribute to pathology (98). In fibrosis, post-translational modifications of matrix proteins may also contribute to the reduced turnover of the proteins and to other pathological effects, e.g. increased tissue TGM2 activity promotes cross-linking of collagen, which not only reduces turnover but also increases the stiffness of the matrix (12, 99) which, in turn, induces changes in cell behaviour. For example, in fibroblasts seeded in hydrogels, increased stiffness of the matrix stimulates proliferation and stress fibre formation (100) both characteristics of fibrosis. Another collagen cross-linking enzyme, lysyl oxidase, is also upregulated resulting in a highly crosslinked collagen network which similarly contributes to TI fibrosis (101). In addition to the accumulation of ECM proteins, non-protein components also
accumulate in the matrix in CKD. Increased HA production was observed in vitro and (human proximal tubular cells and, rat interstitial fibroblasts exposed to high glucose) and in vivo models of diabetic nephropathy (102-105). A significant increase in interstitial HA was also reported in the kidneys of patients with diabetic nephropathy compared with healthy controls (106). In a rat model of CKD (induced by cyclosporine A), increased levels of HA and its receptor (CD44) were observed in fibrotic areas of the interstitium (107). Lipids are not normally present in the ECM of the healthy kidney. However, in diseases such as diabetic nephropathy, circulating lipid particles (eg. blood lipids) are deposited in the matrix (64) with accumulation of lipid evident in both glomeruli and the interstitium in patient biopsies (108). Accumulation of lipids in the renal ECM has also been reported in C57BL/6J mice fed a high fat diet and rat with puromycin aminonucleoside-induced nephrosis (109-111). It has been shown that both native and glycated low density lipoprotein (LDL) can be oxidized by mesangial cells (108) and oxidation of lipids also reduces cellular uptake leading to accumulation of lipids in the ECM during hyperlipidemia (108). Further, other researchers have shown that oxidized LDL stimulates human mesangial cells to produce TGF-β and matrix proteins, such as collagen I, III, and IV (112). Taken together, it might suggest that LDL can be trapped and accumulate in the ECM and promotes fibrosis in patients with hyperlipidemia in CKD (109).

In addition to increased deposition and post-translational modification of the ECM in fibrosis, there are alterations in the composition, for example, foetal isoforms of ECM proteins reappear (12, 113). For example, LAM α5β2γ1 is present in mature GBM while LAM α5β1γ1 is only present in the GBM during development. In Lamβ2 global knock out mice, LAM β1 chain reappears in the GBM resulting in podocyte abnormalities (foot process effacement and loss of the slit diaphragm) and proteinuria (37). In Tgf-β1 transgenic mice overexpressing active TGF-β1, foetal isoforms of LAM (α1, α2, and β2) were detected and result in thickening of the GBM (114). The distribution of ECM components is also altered during fibrosis, for example, interstitial collagen I (115) is
present in the BM and the BM collagen IV in the interstitial matrix (116, 117). Such alterations in the ECM likely lead to changes in the phenotype and function of cells interacting with the matrices (118). For example, collagen IV is required for cell polarisation (119) and collagen I would not provide such information (119, 120). The changes of collagens in the BM could be sensed by epithelial cells via cell surface receptors, such as integrins and DDRs, and result in loss of cell polarity and change in shape (120).

Changes of the mechanical and compositional properties of ECM are sensed by cells via transmembrane matrix receptors such as integrins, DDRs and syndecans (discussed in Section 1.1.2). A number of integrins have been implicated in fibrosis. The collagen-binding integrins α1β1 and α2β1 are believed to play an essential role in fibrosis (121). Studies have shown that integrin α1β1 negatively regulates collagen gene transcription (122), whereas integrin α2β1 positively regulates collagen synthesis (123), however, increased expression of both integrins α1β1 and α2β1 was reported in human kidney interstitial fibrosis and a human renal interstitial fibroblast cell line (Tk188) (124, 125) which may suggest either that the profibrotic effect primarily acts on integrin α2β1 (stimulating collagen production) and there are other integrin-independent mechanisms involved in interstitial fibrosis (126-128). The αvβ3 and αvβ5 integrins have been implicated in myofibroblast differentiation. Blocking both αvβ3 and αvβ5 simultaneously suppressed TGF-β1-induced α-SMA expression and contraction in human cardiac fibroblasts in vitro (129), however, blocking either integrins alone results in only partial reduction in α-SMA expression. In addition, in vivo, the global deletion of only Itgβ3 in mice has no effect on the response to UUO (130), illustrating that, in vitro and in vivo, both integrin αvβ3 and αvβ5 need to be blocked for suppression of myofibroblast differentiation or fibrosis, and these integrins are potentially important in the basic mechanism of fibrosis in a number of different contexts. The αvβ6 and αvβ8 integrins are involved in regulating TGF-β bioactivity (14, 121) and have distinct effects (14, 121). For examples, in Col4A3\textsuperscript{-/-} mice (mimicking Alport syndrome; global germ-line knock out),
the αvβ6 integrin expression is increased and correlated with the progression of fibrosis in the kidney, (131) while blockade of αvβ6 by monoclonal antibody leads to reduced expression of TGF-β1 in the kidney of Col4A3−/− mice (131). In contrast, deletion of αvβ8 integrin rat mesangial cells in vitro results in more active TGF-β (132, 133). These examples illustrate the complex roles of different integrins in renal fibrosis. The DDRs have also been implicated in fibrosis (18). For example, in the murine UUO model, increased expression of DDR1 was detected in the TI of the obstructed kidney (134), and deletion of the DDR1 gene (global germ-line knock out) reduced expression of inflammatory cytokines, TGF-β and fibrillar collagen (134) suggesting the involvement of DDR1 in renal fibrosis. Syndecans are another group of transmembrane matrix receptors which are associated with fibrosis. For instance, in patients with type II diabetic nephropathy, increased levels of syndecan-2 were reported (23). In addition, in aristolochic acid-induced nephropathy, less collagen accumulation and less extracellular TGM2 was observed in syndecan-4-knock out (global) mice compared with WT controls suggesting that syndecan-4 is involved in the externalization of TGM2, which leads to crosslinking of ECM in fibrosis (28, 135)

Fibrotic changes are also apparent in normal ageing of the kidney (12, 136). For example, in 24 month-old rats (equal to 60 years old in human), expression of the LAM β1 chain is up-regulated in the thickened GBM, and there is an increase in interstitial FN compared with 6 month-old animals (equal to 18 years old in human) (137). However, much of the available data on the matrix of normal kidney and age-related changes in the renal ECM are from candidate-based approaches, and a detailed comparison of the composition of the ECM of the TI and the glomerulus in normal, aged or diseased human kidneys using non-biased omic techniques has yet to be performed.

1.2 Proteomics

Much of our understanding of the ECM composition in health and disease has come from assessing the expression of candidate molecules using techniques such as
IHC and Western blotting (138). This type of approach can only examine a relatively small number of selected proteins and is dependent on the availability of reagents for target detection. Therefore, an alternative non-biased high capacity approach is required to profile a wider range of differences in ECM composition in healthy and diseased organs. Proteomics provides a non-biased high capacity method with the added advantage of potentially revealing post-translational modifications of matrix proteins (139) and when combined with bioinformatic techniques, can reveal potential protein-protein interactions in the ECM (140).

1.2.1 Definition of the proteome

In the 1990s, Wilkins first used the term “proteome” to describe the complement of proteins expressed by a genome (141). The Human Genome sequence data showed that humans have 19,599 genes (142) which encode proteins, but modifications in DNA transcription and translation such as alternative splice products and post-translational modifications could result in the expression of 5 times more proteins or fragments (143). Therefore, the level of complexity of the proteome is much higher than that the genome. Currently, a proteome is described as all the proteins expressed by a defined cell type or organism under specific conditions over a certain time period (143, 144).

1.2.2 Development of proteomics

Over time several different methods have been adopted for proteomic studies (145). There has been progressive development from looking at multiple proteins using array-based methods to more complex techniques, such as mass spectrometry. Early array-based proteomic methods emulated the gene microarray platform. Small probes, peptides or antibodies, which bind to the target protein, were fixed on a 2-dimensional (2D) surface, such as a nitrocellulose membrane or glass slide, in known positions to create an “array”. The experimental sample was then incubated with the array to allow interaction between the probe and targeted protein and this interaction was detected as a signal by pre-labelling the experimental sample with a fluorescent probe (145). Two-dimensional electrophoresis in SDS-PAGE gels was also used to study the proteomes.
of bacteria, cultured cells and tissues (146). Proteins were separated based on their isoelectric point and mass (147), and the gel stained with Coomassie blue or silver nitrate to generate a reference map (146). Proteins were then excised from the gels, digested and put through the mass spectrometer for protein identification (146, 148).

The concept of mass spectrometry (MS) dates from the 1890s, and its application was largely limited to the field of physics and chemistry (149). During the development of MS in the early 2000s, several ionisation techniques were invented, including electrospray ionisation (ESI). ESI enabled the analysis of liquid samples (containing proteins up to 1000 kDa), and the introduction of ESI allowed a combination of liquid chromatography (LC) and ionisation leading to the development of LC-MS/MS. LC-MS/MS linked with bioinformatics can be used to analyse a wide range of biological samples (140, 148, 150, 151), and was also adopted in this thesis as this technique had previously been used to analyse the human glomerular ECM (139).

1.2.3 Application of proteomics to the analysis of ECM

1.2.3.1 Use of proteomics for the analysis of ECM from cells and organs

Proteomics can be used to analyse the matrix from cultured cells and simple-structured tissues (152-154) and provides insights into the complexity of the composition of the ECM produced by different cell types. For example, proteomic analysis of the matrix produced by the human hepatic stellate cell line LX-2 (a model of liver fibrosis) identified 250 proteins of which 48 were identified as matrix-related proteins using UniProt (153). In addition, proteomics can identify novel protein in the matrix produced by cells (153). For example, in the study mentioned above, two proteins, CYR61 and Wnt-5a were shown in the matrix of LX-2 cells, and had not been reported as increased expression in the fibrotic liver matrix previously (153).

Proteomics can also be used to characterise the matrices of simple-structured tissues which are much more complicated than those of cultured cells, and identify novel matrix proteins. For instance, proteomic analysis of the human aortic matrix using ECM enrichment (139) in combination with MS-based proteomics, identified 103 matrix
proteins. Among them, podocan, sclerostin, and agrin were identified in the human aortic matrix for the first time at protein level (155). Matrix from more complex organs of normal and diseased animals can also be analysed by proteomics and reveal disease-related changes in the matrix. As an example, analysis of matrix isolated from pancreatic islets of a mouse insulinoma model and controls revealed a total of 120 proteins (combining the control and insulinoma groups; it should be noted that in proteomic studies many investigators combine and present the total number of proteins from all samples as a measure of the total number of proteins that were identified (140, 148, 150, 151)). Of these 120 proteins, 35 were significantly changed (9 increased and 26 decreased) during the insulinoma development (154).

Proteomics can also be applied to analyse samples from complex organs containing different tissues architecture with multiple cell types and their associated ECM (140, 156). For example, MS-based proteomic analysis of whole mouse lung tissue and bronchoalveolar lavage fluid after bleomycin-induced lung injury compared to normal controls, identified a total of 8336 proteins of which 435 matrisome proteins were identified using the mouse matrisome database (140, 157). Additionally, proteomics can be used to identify disease-related changes and potentially to identify novel targets for therapy. Analysis of samples (above study) from different time-points post-injury (3, 14, 28 and 54 days) revealed 154 matrisome proteins that showed significant changes in at least one time-point. Among these proteins, collagen XXVIII and Emilin-2 were highlighted as potential contributors in the progression of lung fibrosis for the first time, which may represent new targets for anti-fibrotic therapy development (140). Proteomics has also been employed to analyse decellularised tissues (the technique of decellularisation is discussed in Section 1.3) from normal and diseased tissues to identify disease-related changes in the matrix. For instance, proteomics of samples from decellularised normal and fibrotic (idiopathic pulmonary fibrosis (IPF)) human lung (158) identified 94 matrix proteins in the normal lung compared with 85 proteins identified in IPF of which 7 proteins were only seen in the fibrotic samples, and 16 proteins were
unique to the normal samples. Of the shared proteins, 17 proteins increased at least 2-fold in IPF compared with normal and 22 proteins decreased by at least 50% (158). In addition, the ECM of human liver and heart have also been analysed by proteomics to identify disease-related changes. For example, proteomics of decellularised biopsies from patients with liver fibrosis (Hepatitis C infection-induced) showed the association of 47 matrix proteins with progression of liver fibrosis (159). In decellularised cardiac tissue from dilated cardiomyopathy (DCM) patients and normal hearts, a total of 1133 proteins (combining the DCM and normal samples) was identified by proteomics, of which 75 showed significant differences. Twenty-six of those 75 proteins were structural proteins, of which 11 proteins showed increased expression in DCM group and 15 decreased (160). Taken together these studies show that proteomics provides a powerful tool to reveal the complexity of matrix from cell to complex organs, and examine changes in matrices of simple and more complex tissues in health and disease.

1.2.3.2 Proteomic analysis of whole tissue and ECM from kidney

Although the kidney has a number of different tissue compartments (161), the majority of the studies have profiled the whole ECM (as reviewed by Stojnev and colleagues (162, 163)). Where studies have examined the ECM of particular tissue compartments, most have focused on the cortex (139, 164-170), with very few looking at the medulla (166, 169, 171) and even fewer separating medulla and papilla (172). Proteomics has been used to analyse protein expression in the matrix generated by kidney cells in vitro (human podocytes and glomerular endothelial cells) (34), in kidney tissues from ageing and diseased animals such as mice, rats, monkeys and marmosets (81, 173-178), in the matrix of normal whole human kidney (162, 168) and in normal human kidney glomerular matrix (139, 167).

1.2.3.2.1 Proteomics of the normal kidney ECM

In the cortex of the kidney, there are 2 major tissue compartments, glomeruli and TI (Figure 1.1). The normal human glomerular ECM was analysed using MS-based proteomics (139). Glomeruli were isolated by sieving to enrich for glomerular proteins
and subjected to fractionation to enrich for ECM proteins prior to analysis. One hundred and forty-four glomerular ECM proteins were identified, which included all previously identified glomerular-related proteins such as collagen VI α1 and LAM α5 as well as numerous new components including latent-TGFβ-binding protein 1 (LTBP1) and collagen IV α6. Protein networks were analysed using an in-house matrisome database, and relative quantification between different samples revealed a core connected network of 48 structural ECM proteins suggesting that proteomics combined with bioinformatic techniques can reveal the composition of the matrix in human kidney and provide insights into the complex protein-protein interactions within the ECM. In another study, laser capture microdissection in combination with MS was used to analyse glomeruli from human kidney sections (167). This study identified a similar number of ECM proteins (147) as reported by Lennon et al. (139). Together these proteomic studies demonstrated that the composition of the glomerular ECM is more complex than had previously been envisaged.

Using a similar MS-based proteomic approach, Lennon and colleagues further compared the composition of the ECM of glomeruli in vivo (139) and ECM produced by glomerular cells in vitro to understand how different glomerular cell types contribute to the GBM (34). ECM isolated from mono-cultures and co-cultures of immortalised human glomerular endothelial cells (GEnC) and podocytes were analysed using in-gel digestion and LC/MS-MS. Distinct cell type-specific ECM was identified in mono-cultures (127 proteins identified in GEnC ECM and 142 in podocyte ECM with 95 shared proteins), in contrast, the ECM from co-cultures of the two cell types showed a different ECM organisation and composition (123 ECM proteins identified) and the formation of a BM-like structure between two cell types. These data demonstrated that co-culture of cells produce different matrix compared to individual mono-cultures suggesting that intercellular communication/interaction is important in determining the final matrix composition. Comparison of results from mono- and co-culture with glomerular ECM proteomics followed by protein network analysis identified 35 shared structural proteins.
in all four ECMs (mono-cultures, co-culture and intact glomerular ECM) that are likely to have important roles in the glomerular ECM (34). Co-culture of podocytes and GEnCs produced a matrix more similar to human glomerular ECM (formed BM-like structure with 51 ECM proteins shared with human glomerular proteome (144 proteins)) (34, 139) and demonstrated the utility of proteomics to provide insights of how different cell types individually and in combination, produce unique matrices. Gender and strain specificity of glomerular ECM composition and disease susceptibility has also been studied by comparing two mouse strains, FVB and B6 (174) (FVB mice are more susceptible to disease and develop more severe disease than B6 mice in the models of kidney disease (174, 179-183)). The investigators found that adult FVB mice had an 8-fold increase in albuminuria compared with age-matched B6 mice, and 2-fold greater albuminuria in males of each strain compared to females. Proteomic analysis revealed that, in the glomerular ECM, the expression of Fgf-2, netrin 4, and meprin 1-α and 1-β was positively correlated with high albuminuria while there was a negative correlation with collagen I and tenascin C expression. In this study, proteomics provided insights into differences between matrices in kidneys of animals with different levels of proteinuria and gave potential clues for the susceptibility of disease (174). Taken together the studies outlined above show that proteomics provides a powerful tool to analyse the ECM in detail and identify proteins not previously described in the matrices examined (34, 139, 167, 174).

In addition to the glomerular ECM, the human kidney cortex also contains matrices within the TI (tubular basement membranes, capillary BM, vessel walls, and interstitial matrix). In contrast to the glomerular ECM, relatively little is known about the global composition of the cortical TI ECM. This is perhaps surprising, given that accumulation of ECM in the TI provides the best prognostic indicator to end-stage renal disease in CKD (184). However, to date, no study has examined the TI ECM using non-biased proteomic approaches.

1.2.3.2.2 Applications of proteomics to the analysis of the ageing kidney
In the kidney, ageing leads to significant functional and structural changes (136) with the appearance of fibrosis, inflammation and changes in vascular tone leading to an age-related decline in renal function (136, 185, 186). Although as discussed in Section 1.1.3.1, candidate-based approaches have been applied to study the ECM of the ageing kidney, there have been relatively few studies that have addressed ageing in the kidney using proteomics. There are a few studies which have used proteomics to investigate age-related changes in the whole kidney tissue from a variety of species. Comparison of 13 and 31 month-old male Fisher 344 rat kidneys (equal to 32 and 77 years old, respectively) by 2D gel electrophoresis identified 164 spots which were differentially expressed and of those MS identified 103 proteins that were significantly altered (2-fold increase/decrease in expression) in the ageing kidney, for example, cathepsin B and glutathione peroxidase both increased with age (177). Another study revealed age-related changes in male and female CD1-Swiss mice using 2D gel electrophoresis and MS. Comparing kidneys of 28, 52 and 76 week-old mice (equivalent in human age: 38, 58 and 74), 22 proteins (spots) were altered in both 52-week (13 spots down-, 9 up-regulated) and 76-week (9 down- and 13 up-regulated) male mice compared to 28-week mice and 33 in ageing females (14 down- and 19 up-regulated in the 52-week and 13 down- and 20 up-regulated in the 76-week mice) (173). Mice bearing Klotho gene mutations which lead to premature ageing resembling Progeria (accelerated ageing) in humans, have also been used as a model to study age-related changes. Proteomic analysis of kidneys from Klotho mutant (global knock out) and WT mice using matrix-assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS) and LC/MS-MS, identified 97 proteins in WT and 69 proteins in mutant kidneys (33 proteins identified in both groups; the relatively low number of proteins identified may be due to the fact that 10µm frozen sections were used). The most abundant proteins (62% in WT and 65% in mutant kidneys) were nucleic acid-binding proteins and enzymes with only a few matrix proteins identified such as nidogen-1 (187). These studies demonstrate that proteomics can provide an in-depth analysis of changes in protein expression in ageing
tissue. However, the studies did not separate/enrich matrix from total tissue which might affect the identification of matrix proteins as they are relatively low abundant compared with cytoplasmic proteins. These studies have used tissue from animal models rather than from humans. As changes in the ECM in ageing kidney likely contribute to the functional changes of kidney in ageing, there is a need for more information about how the human kidney ECM changes with age.

1.2.3.2.3 Validation of proteomics

Like all array techniques, proteomics can be subjected to false discovery and it is important to validate the results using a second method (eg. candidate-based techniques). The most commonly used validation methods are IHC, IFL and Western blotting and each method has its advantages and disadvantages. IHC and IFL can localise the expression of protein to particular structures, while Western blotting is more appropriate for relative quantification. For example, IHC and IFL were used to confirm the expression of collagen VI in a glomerular cell co-culture system (34) and the expression of elastin in the vessels of diabetic patients (127). Western blotting was used to confirm some of the matrix proteins identified in normal human glomeruli (139) and to confirm alterations in matrix proteins in mice with different levels of albuminuria (174).

Taken together the studies described above provide clear evidence that proteomics is a powerful tool for global protein identification with broad applications providing comparisons of changes in protein expression in a wide range of settings including in cells in culture, in normal tissues and in particular in tissues in ageing and disease.

1.3 Tissue decellularisation

Another approach to study the matrix is to decellularise tissues or organs, which enables researchers to look at the matrix in the absence of cellular components. Decellularisation can provide a number of benefits: 1) it allows characterisation of the matrix without potential masking by the cellular components; 2) it provides a platform to
study cell-matrix interactions; 3) it can provide a scaffold for use in regenerative medicine to understand how to create new organs.

1.3.1 History of decellularisation

More than 40 years ago, Meezan and colleagues used chemical and enzymatic treatment (4% sodium deoxycholate (SDC) and 2000 Kunitz Units DNase I) of rabbit renal tubules and bovine retinal and brain blood vessels to remove the cellular components and to characterise the ECM by electron microscopy (188). During the 1970s and 80s, decellularisation (using a variety of protocols) was used to study a variety of ECM, for example, bovine glomeruli (189) and guinea pig trachea (190). In the 1990s and 2000s, decellularisation of tissues such as the trachea (guinea pig), bladder (rat) and sections of blood vessels (rat), was achieved for tissue regeneration(189, 191). In 2008, Ott and co-workers developed the first method to decellularise a whole organ (rat heart) by perfusion for use in studies of organ regeneration (192). Over the last decades, decellularisation studies have mainly been in the context of tissue engineering (189) driven largely by the increasing demand for organs for transplantation (193) and the scarcity of donated organs. In the UK alone, there are 49,000 patients waiting for an organ transplant (https://www.organdonation.nhs.uk/). As a result, there have been intensive attempts to create artificial organs/tissue for transplantation (194). Although synthetic organs could potentially be created using biocompatible materials combined with 3D printing or casting (195), there are significant challenges in replicating the complex components and ultrastructure of native tissues. This has led to an increasing interest in decellularising organs to generate non-immunogenic biological scaffolds (194). Therefore, many decellularisation studies have focussed on decellularising whole organs to provide a scaffold on which to reseed cells such as induced pluripotent stem cells (iPSCs), with the aim of potentially creating functional organs for patient-specific transplantation (196). While tissue regeneration has been the main driver for the development of decellularisation techniques the scaffolds produced also provide an
opportunity to study the composition and structure of the ECM devoid of cellular components and to understand how the ECM regulates cell behaviour,

1.3.2 Whole organ decellularisation

The aim of decellularisation is to remove cellular components while preserving the ECM composition and ultrastructure. However, it is challenging to achieve both simultaneously and a balance between the preservation of ECM structure and removal of cellular components has to be achieved. Decellularisation of different organs has been achieved by a variety of different methods, and a number of studies also considered changes in the mechanical/physical properties of decellularised scaffolds compared to native tissue as these changes could affect cell behaviour (194, 197, 198). Tissues that have been successfully decellularised can be broadly divided into two categories: small/simple-structured tissue eg. blood vessels, muscles and small intestine, and large/complex organs eg. lung, liver and kidney.

1.3.2.1 Decellularisation of simple-structured tissues

In terms of the vasculature, equine carotid and porcine arterial segments were decellularised using a mixture of 0.5% SDS (sodium dodecyl sulfate) and 0.5% SDC followed by 125 U/mL endonuclease treatment over 3 days (199) or using 4% SDC followed by 2000kU DNase I over 4.25 days, respectively (200). The removal of cellular components was confirmed by haematoxylin and eosin (H&E) staining and DNA quantification. In addition, Pellegata and colleagues used scanning electron microscopy (SEM) to demonstrate that the luminal surface of vessels was intact after decellularisation (200), indicating effective decellularisation with the different detergents. In addition, pig aortic valves and heart valve leaflets were successfully decellularised by incubation for 2 days in 0.05% trypsin with PBS washing (shaking) and 1% SDS with PBS washing (shaking), respectively (201, 202). In the trypsin-treated heart valve ECM, there was some minor loss of elastin while the amount of collagen was the same as in native tissue (201). Two-photon laser scanning fluorescence microscopy showed intact but compact collagen fibres in the SDS-treated leaflets (202) suggesting that the
detergent might slightly alter the matrix structure. Together these studies suggest that different detergents differentially affect vascular matrix proteins during treatment and potentially alter the matrix structure. Therefore, detergents for organ decellularisation should be carefully selected and may require optimisation.

In addition to detergents and enzymes, non-polar supercritical CO$_2$ has been used for tissue decellularisation due to its unique physical properties (density like liquid/diffusivity like gas/7.4MPa critical pressure/31.1°C critical temperature) (203). Supercritical CO$_2$ fluid mixed with ethanol was used to decellularise pig aorta (203). Ethanol was added to dissolve the polar cellular material such as cell membrane components and DNA. Cell nuclei were successfully removed by a 15-minute treatment at 37°C with preservation of matrix components such as collagens and elastin, although phospholipids could not be completely removed even with prolonged treatment (203). Interestingly, physical pressure can also be used to facilitate decellularisation (204). Porcine aorta was cut into 10 x 2mm pieces and packed in a plastic bag filled with PBS. A pressure of 980 MPa was applied for 10 minutes at 10 or 30°C, with a rate of increase of 65.3 MPa/minute and decrease of 196.1 MPa/minute. Samples were then washed with PBS for 14 days. It was found that due to the high pressure, ice formed in samples treated at 10°C, resulting in ultrastructural damage and loss of collagen fibres, while at 30°C the ultrastructure and collagen fibres were retained with more than 95% DNA removed and no cell nuclei observed in H&E-stained sections (204). Although an apparent advantage of using high pressure and supercritical CO$_2$ is a marked reduction in the time required to disrupt cells, the total time for decellularisation was not reduced compared with detergent-based methods due to the extensive washing steps required. Moreover, there are no reports of the use of high pressure and supercritical CO$_2$ to decellularise whole organs possibly due to the complexity of tissue structure, suggesting this approach has not been widely adopted.

Other simple-structured tissues that have been successfully decellularised include trachea, small intestine and bladder. Human, pig and rat trachea have all been
decellularised using 25 (human and pig) and 6 (rat) cycles of 4% SDC, DNase I (2000KU/ml) for a total of 25 days and 6 days, respectively. Although DNA content was reduced by 99% in human, 65% in pig and 90% in rat, DAPI staining showed some residual cells in the cartilaginous regions of all the tissues (205-207). In the pig trachea, despite the use of DNase and a 65% reduction in DNA content, DAPI staining showed that nucleic acids accumulated along ECM fibres (207). Surprisingly, even though same decellularisation protocol was used, there appeared to be significant difference observed in removing DNA between human and pig trachea and the reason for this observation is unclear (207), therefore, optimisation for particular organs in different species is essential. The decellularisation protocols tested had no effect on the amount of elastin remaining in the decellularised scaffold compared with native tracheal tissue, and SEM showed good ultrastructural preservation (205-207). Despite the good preservation of matrix, the investigators suggested further optimisation is required for complete decellularisation of the trachea as nucleic acids accumulated along ECM fibres in all species. In a later study, rat trachea was decellularised with 3 cycles of 22 hours in 8mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 minutes PBS wash, 22 hours in 1.8mM SDS, 30 minutes PBS wash (208). Histology and DAPI staining showed complete removal of nuclear and cellular materials and DNA quantification confirmed the elimination of more than 99% of DNA (208). Comparing this study with previous ones suggests that, different types of detergents, such as ionic and non-ionic detergents, may be required for complete decellularisation of trachea. Another tubular tissue that has been decellularised is the small intestine. Pig small intestine was decellularised using different methods (197). Three combinations of chemical/enzymes (1% SDS/1% Triton (12.5 hours); 4% SDC, DNase I 6.66KU/ml (16 hours); or 0.1% Peracetic acid (PAA) (12 hours), were compared (197). The SDS/Triton-treated group showed the best DNA and cell removal (confirmed by H&E staining and DNA quantification) with good ultrastructural preservation (using SEM). In mechanical tests, scaffolds decellularised with SDS/Triton or SDC/DNase were similar while those decellularised with PAA showed
an increased elastic modulus and calculated yield stress compared with all other groups suggesting that PAA-treated scaffolds are stiffer (197). A similar observation was reported in porcine urinary bladder decellularised by immersion in 0.1% PAA for 3 hours. The preservation of ultrastructure was confirmed by SEM, and the increase in stiffness was attributed to changes in collagen fibre alignment (198, 209). Overall, the decellularisation of tissue with a relatively simple structure is feasible, however, the procedure for decellularisation is highly variable for different tissues and even for the same tissue from different species thus there is a requirement for careful optimisation of protocols.

### 1.3.2.2 Decellularisation of more complex organs

More complex organs such as heart, lung, liver and kidney, can also be decellularised either by perfusion of the whole organ or by cutting the tissue into pieces (194, 210-214). Whole rat heart was decellularised by perfusion with 1% SDS for 6 days (192) with good preservation of matrix components (LAM, Collagen I and III and FN; confirmed by IFL) and ultrastructure (SEM) (192). Similarly, perfusion was used to decellularise rat, porcine and human lungs (210). For the rat lung, three protocols were compared: 0.1% SDS, 8mM CHAPS and 2% SDC perfusion (for around half a day with the duration determined by visual assessment of tissue clearing), and showed similar DNA removal, however better preservation of collagens was observed in the SDS-treated samples (210), the investigators did not provide any explanation for this observation. The investigators also reported that perfusion decellularisation of porcine and human lung required 6-9 days treatment with 0.5% SDS with daily changes of solution. H&E confirmed cell removal and transmission electron microscope (TEM) revealed good preservation of ECM ultrastructure (210). As well as whole organs, 2mm thick sheets of human and pig lung have been decellularised by incubation in 1.8mM SDS or 8mM CHAPS for 14 hours. The collagen content of the scaffolds was similar with the two detergents and good preservation of the ultrastructure was confirmed by SEM (212) suggesting that there is no advantage to one detergent over another in this
experimental setting. Decellularisation of liver has also been achieved (213-215). Whole normal and fibrotic (carbon tetrachloride-treated) rat livers were perfused with 0.02% trypsin, 0.05% EGTA for 1 hour followed by perfusion with 1% Triton, 0.05% EGTA for 36 hours and samples washed with PBS containing 1% PAA. Removal of cells was confirmed by H&E with a 90% reduction in DNA content. As expected, there was an increase in Sirius Red staining in fibrotic tissue scaffolds with an increase in collagen I, LAM β1 and FN confirmed by IFL (215) indicating that differences in normal and fibrotic ECM are preserved after decellularisation. Although detergents are frequently used and can achieve good decellularisation, almost inevitably there is some loss of the matrix components which varies depending on the organ decellularised (213, 214). For example, Park and colleagues compared 5 different protocols (0.1% SDS, 1% SDS, 4% SDS, 1% Triton X-100 and 1% Triton with 1% SDS) to decellularise pig liver cubes (1x1.5x0.5cm) and showed that complete decellularisation could be achieved with 0.1% SDS (2.13 days) with the less damage to the matrix (60% of total collagen content was preserved) than with the other solutions (214). Although better collagen preservation was reported in the Triton only and Triton plus SDS treated tissues, residual cellular material was observed, suggesting that loss of collagen might be inevitable in removing all cellular material. In a later study, pig liver cubes (1x1x0.5cm) were decellularised in 0.1% SDS for 3 days and washed with 0.1% PAA for 30 minutes (213). Removal of the cellular component was confirmed by H&E with elimination of more than 99% of DNA. Although total collagen content (the level of hydroxyproline) was reduced to 65%, SEM showed that the ultrastructure was largely preserved, and intact collagen fibres were observed by TEM. This may suggest that during decellularisation some collagens were removed although fibrillar collagens remained, highlighting the need to reduce the length of SDS treatment to prevent loss of ECM components. Interestingly, the same combination of chemicals was used to decellularise whole rat livers by perfusion via the renal artery, over 34 hours. Similar removal of cellular components was achieved but with no reduction of total collagen content (213). It may be speculated that the difference between the level of
collagen preservation in decellularising whole rat liver compared with pig liver cubes is due to the reduction in time of SDS treatment and less exposure of the matrix to SDS in perfusion decellularisation although species differences cannot be excluded. In summary, taken together these studies showed that both perfusion- and cube-based decellularisation of more complex organs is achievable, and even in the same organ decellularising cubes is different from decellularising whole organs and optimisation is required during the development of decellularisation.

1.3.2.3 Decellularisation of the kidney

Kidneys from a variety of species including pigs, rats, mice, monkeys and humans have been decellularised and protocols have been optimised by considering different variables, including the choice of chemicals and enzymes and the duration of each step (Table 1.1). Rodent kidneys and in particular rat and mouse kidneys, were most commonly used in the early studies due to the ready availability of these organs (193, 196, 216-219). The architecture of pig kidneys is more similar to human kidney, and an increasing number of studies have used pig kidneys for decellularisation as these are also relatively easy to obtain (220-223). A limited number
<table>
<thead>
<tr>
<th>Species (Reference)</th>
<th>Size of tissue (whole organ/ slices/ cubes)</th>
<th>Chemicals/detergents/enzymes</th>
<th>Total time for decellularisation</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(224)</td>
<td>Whole</td>
<td>1% SDS, 1% Triton</td>
<td>20 hours (H)</td>
<td>H&amp;E, Masson’s Trichrome, SEM and IFL</td>
</tr>
<tr>
<td>(225)</td>
<td>Whole</td>
<td>1% SDS</td>
<td>8H</td>
<td>H&amp;E</td>
</tr>
<tr>
<td>(217)</td>
<td>Whole</td>
<td>0.1% Triton, 0.8% SDS</td>
<td>8.3 days</td>
<td>H&amp;E, Masson’s Trichrome, Periodic acid–Schiff (PAS), TEM, vascular corrosion casting, IFL</td>
</tr>
<tr>
<td>(218)</td>
<td>Whole</td>
<td>1% SDS</td>
<td>20H</td>
<td>H&amp;E, IFL, TEM, SEM, SEM, Micro-CT</td>
</tr>
<tr>
<td>(226)</td>
<td>Whole</td>
<td>3% Triton, 0.0025% DNase I, 3% Triton, 4% SDS</td>
<td>5 days</td>
<td>SEM, H&amp;E, IHC, IFL</td>
</tr>
<tr>
<td>(227)</td>
<td>Whole</td>
<td>0.5% SDS</td>
<td>28H</td>
<td>H&amp;E, TEM, IHC, fluoroangiography, DNA and total collagen quantification</td>
</tr>
<tr>
<td>(193)</td>
<td>Whole</td>
<td>Triton + SDS, 0.0025% DNase I (detergent concentrations not reported)</td>
<td>5 days</td>
<td>H&amp;E, IFL</td>
</tr>
<tr>
<td>(216)</td>
<td>Whole</td>
<td>1) 0.125% SDS</td>
<td>1), 2) and 3) 32H</td>
<td>H&amp;E, IHC, DNA quantification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 0.25% SDS</td>
<td>4) 4H</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 0.5% SDS</td>
<td>5) 2.3 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4,5) 1% SDS (short or long treatments)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(196)</td>
<td>Whole</td>
<td>1) 1% Triton</td>
<td>1) 27H</td>
<td>H&amp;E, SEM, IHC, IFL, vascular corrosion casting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 1% Triton, 0.1% SDS</td>
<td>2) 27H</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 0.02%Trypsin, 1% Triton</td>
<td>3) 26H</td>
<td></td>
</tr>
<tr>
<td>(219)</td>
<td>Whole</td>
<td>1% SDS, 1% Triton</td>
<td>4.5 days</td>
<td>H&amp;E, IFL, TEM, IHC, DNA and total collagen quantification</td>
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<td>(228)</td>
<td>Whole</td>
<td>1% SDS, 1% Triton</td>
<td>9H</td>
<td>H&amp;E, IFL, mass spectrometry, DNA quantification</td>
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<td><strong>Pig</strong></td>
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<tr>
<td>(229)</td>
<td>Whole</td>
<td>SDS (concentration not reported)</td>
<td>6.5 days</td>
<td>H&amp;E, Masson’s Trichrome, IFL, SEM. fluoroangiography</td>
</tr>
<tr>
<td>(221)</td>
<td>Whole</td>
<td>1) 0.5% SDS, 0.0025% DNase</td>
<td>1) 4 days</td>
<td>H&amp;E, DNA quantification, SEM fluoroangiography, vascular casting,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 1% Triton, 0.5% SDS</td>
<td>2) 6 days</td>
<td></td>
</tr>
<tr>
<td>(230)</td>
<td>Whole</td>
<td>0.5% SDS, 0.0025% DNase</td>
<td>36H* (SDS)</td>
<td>N/A</td>
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<tr>
<td>--------</td>
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</tr>
<tr>
<td>(231)</td>
<td>Whole</td>
<td>0.5% SDS, 0.0025% DNase</td>
<td>36H* (SDS)</td>
<td>N/A</td>
</tr>
<tr>
<td>(223)</td>
<td>Whole</td>
<td>1) 1% SDS&lt;br&gt;2) 1% Triton&lt;br&gt;3) 1% PAA&lt;br&gt;4) 1% SDC</td>
<td>36H H&amp;E, IFL, SEM, Masson’s Trichrome, Sirius red, DNA and total collagen quantification</td>
<td></td>
</tr>
<tr>
<td>(220)</td>
<td>Slices (7mm diameter 2mm thickness)</td>
<td>1) 0.1M NaOH&lt;br&gt;2) 1% PAA&lt;br&gt;3) 3% Triton&lt;br&gt;4) 1% SDS&lt;br&gt;5) 0.05% Trypsin/EDTA</td>
<td>More than 10 days H&amp;E, SEM, DNA and total quantification; Sirius red and Orcein elastin staining</td>
<td></td>
</tr>
<tr>
<td>(211)</td>
<td>Slices (2mm thickness)</td>
<td>0.02% Trypsin, 3% Tween, 4% SDC</td>
<td>7.5H H&amp;E, Mason’s Trichrome, Alcian blue staining, SEM, IHC, IFL, DNA and total collagen quantification</td>
<td></td>
</tr>
<tr>
<td>(222)</td>
<td>Cubes (10x10x2mm)</td>
<td>1) 1% Triton, 30µg/ml DNase&lt;br&gt;2) 1% SDS, 30µg/ml DNase</td>
<td>10-14 days H&amp;E, IFL, SEM, Western blotting</td>
<td></td>
</tr>
</tbody>
</table>

**Monkey**

| (232)  | Slices | 1% SDS | 10-14 days | Haematoxylin, IFL, IHC, proteomics |

**Human**

<table>
<thead>
<tr>
<th>(233)</th>
<th>Whole</th>
<th>0.5% SDS, DNase (concentration not reported)</th>
<th>7.75 days IFL, vascular corrosion casting, SEM</th>
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</thead>
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<tr>
<td>(162)</td>
<td>Whole</td>
<td>1% SDS + DNase (concentration not reported), 1% Triton</td>
<td>11 days Haematoxylin, IFL, SEM, proteomics</td>
</tr>
<tr>
<td>(234)</td>
<td>Whole</td>
<td>0.5% SDS</td>
<td>7.5 days H&amp;E, IFL, IHC, SEM, Masson’s Trichrome and Methenamine silver staining</td>
</tr>
<tr>
<td>(235)</td>
<td>Slices (2mm thickness)</td>
<td>0.02% Trypsin, 2% Tween-20, 4% SDC, 1% SDS</td>
<td>Up to 28H H&amp;E, Azan trichrome, PAS and Alcian blue staining, DNA quantification, IFL</td>
</tr>
<tr>
<td>(168)</td>
<td>Cubes (10x10x10mm)</td>
<td>1% SDS</td>
<td>10 days H&amp;E, IFL, proteomics,</td>
</tr>
</tbody>
</table>

**Table 1.1** Summary of published protocols for decellularisation of the human kidney.

+: indicates detergents are mixed; *: total time not reported; SDS: sodium dodecyl sulfate; H&E: haematoxylin and eosin; SEM: scanning electron microscopy; IFL: immunofluorescence; TEM: transmission electron microscope; IHC: Immunohistochemistry; PAA: peracetic acid; SDC: sodium deoxycholate; N/A: not available.
of studies have decellularised primate kidneys (e.g. from Rhesus monkeys) but access to such organs is rare (232). Based on the successful decellularisation of pig and primate kidneys, protocols for the decellularisation of human kidneys have been developed although the availability of organs is generally limited and confined to organs donated for transplantation but which were deemed unsuitable for transplantation (162, 168, 233-235).

Of the different decellularisation methods that have been developed for the kidney, whole organ decellularisation is the most common approach. Almost all protocols for decellularisation of rodent kidneys use whole organ decellularisation where the renal artery, vein and ureter are cannulated and the tissue continuously perfused with decellularisation solutions (193, 196, 216-219). Pig kidneys have also been decellularised by perfusion via the artery and ureter (162, 220, 221, 223).

Alternatives to whole organ perfusion include the use of tissue cubes/slices (2-10mm thickness). Decellularisation of tissue cubes/slices is generally performed by submersion in decellularisation solutions and stirring or shaking at either room temperature or 37°C (211, 220, 222). For example, kidney tissue cubes/slices from pig, monkey and human were decellularised by submersion in SDS for 5-14 days (Table 1.1) (168, 222, 232-235).

1.3.2.4.1 The use of chemicals and enzymes for kidney decellularisation

Many protocols begin by perfusing water through the kidney in order to rupture cell membranes by osmotic pressure (196, 220, 223, 234). Then a variety of chemicals and enzymes have been used individually and in combination, to decellularise kidneys, including Triton X-100, SDS, SDC, sodium hydroxide (NaOH), hydrochloric acid (HCl), DNase and trypsin (Table 1.1). Among them, SDS and Triton are the most commonly used. In a perfusion-based study of decellularisation of whole pig kidney, Wang and colleagues compared 18 hours perfusion using four chemicals individually (1% SDS, 1% Triton X-100, 1% PAA and 1% SDC). The SDS-treated group showed the lowest amount of residual DNA and cytoplasmic proteins in the kidney matrix. Triton-, PAA- and SDC-
treated organs all showed residual cellular components (by histology) and DNA (positive DAPI staining in the matrix), especially in the glomerular structures. SEM of only SDC-treated tissue showed an unusual “wavy” shape of the basement membrane in glomerular structures, suggesting that SDC affects the structure of GBM (223). Caralt and colleagues tested three protocols using Triton X-100 alone or in combination with either SDS or trypsin (1% Triton, 1% Triton/0.1% SDS or 0.02% trypsin/1% Triton) for perfusion decellularisation of rat kidney (196). After 26 hours, 1% Triton and trypsin/Triton failed to remove cellular material with cell debris evident in tubules, glomeruli and vessels. In contrast, Triton/SDS completely removed the cellular components and DNA (as visualised by the absence of DAPI staining). By SEM, a similar level of ECM ultrastructural preservation was observed in blood vessels, tubules and glomeruli following either Triton/SDS and trypsin/Triton perfusion, however, H&E staining showed incomplete decellularisation using Triton alone with some cells observed in the glomeruli (196). These studies showed that successful decellularisation of rat and pig kidneys could be achieved by whole organ perfusion with SDS (alone or in combination with other chemicals/enzymes) with good preservation of glomerular (196), tubular and vascular ECM structures (196, 223). Similar results were obtained by Zambon and colleagues who showed that 4 days perfusion of pig kidneys with 0.5% SDS/0.0025% DNase or 4.75 days with 0.5% SDS/1% Triton X-100 resulted in efficient decellularisation with no ultrastructural damage to the ECM and minimal residual DNA (221). To determine the effect on ECM structure of different concentrations and exposure times of SDS (0.125 - 1%) during decellularisation, whole rat kidney was decellularised by perfusion for 4 - 24 hours (216). This study showed that perfusion with 0.125% SDS for 4 hours was as effective as 1% SDS for 4 hours (216). In summary, although these studies showed SDS is effective in decellularising whole kidneys from pig and rat kidneys, it was also highlighted that the length of time for detergent treatment is also an important variable, which might be associated with the size of decellularised samples and damage of the matrix, suggesting that optimisation of the time for decellularisation is essential to
achieve a good balance between preservation of matrix and efficient decellularisation of kidneys from different species.

Although perfusion decellularisation studies suggested that Triton X-100 is not an effective decellularisation solution for whole pig kidneys, Choi and colleagues (222) showed that complete decellularisation of pig kidney tissue cubes (10 x 10 x 2mm; tissue volume: 200mm$^3$) could be achieved with either 1% Triton X-100 or 1% SDS and shaking for 14 days at 4°C followed by DNase treatment for 1 hour which suggests that the tissue configuration has a significant effect on the efficiency of decellularisation by individual detergents. Both protocols produced a 3D network of matrix devoid of cells and residual DNA. However, it is difficult to conclude which method generated a better matrix scaffold as Triton-treated tissue showed better cytocompatibility (more cells attached to Triton-treated matrix 24 hours after seeding) while the mechanical property (compression strength) of SDS-treated samples more closely resembled the normal tissue (222). A more comprehensive study compared 5 different chemicals, detergents or enzymes individually (0.1M NaOH, 1% PAA, 3% Triton X-100, 1% SDS, or 0.05% trypsin/EDTA) for decellularisation of pieces of pig kidney tissue (2mm thick x 7cm diameter; tissue volume: 308mm$^3$). Tissues were incubated for 20 days (solutions changed daily) with shaking, at room temperature (220). In contrast to the results of the study by Choi et al. (222), Triton X-100, PAA and trypsin/EDTA treatment failed to completely remove cytoplasmic components suggesting that decellularisation with Triton X-100 seems to be very variable and differences in tissue volume, the temperature of incubation and detergent concentration might affect the efficiency of decellularisation. NaOH, PAA and SDS were more effective in removing residual DNA than trypsin/EDTA or Triton, with NaOH treatment resulting in the lowest residual DNA concentration. However, NaOH caused more structural damage (histology showed damaged/shrunk glomerular structures) compared with SDS (220). Therefore, out of the 5 reagents, 1% SDS seemed to provide an acceptable balance between effective removal of cellular components and preservation of matrix structure. Similar results were reported by Nakayama and
colleagues (compared 1% SDS with 1% Triton), who showed that 1% SDS is more
effective in decellularising Rhesus monkey kidney slices with preservation of the tissue
architecture (236).

In summary, in both perfusion- and cube-based decellularisation protocols using
SDS alone or combined with other detergents produced an acceptable balance between
ECM preservation and consistent good tissue decellularisation. However, for kidney
tissue cubes, Triton X-100 alone resulted in either good or incomplete decellularisation
depending on the study, suggesting that Triton X-100 does not achieve consistent
decellularisation and it was not effective for whole organ decellularisation (222). A
number of factors affect the efficiency of decellularisation, including the size of tissue
slices/cubes and the temperature of incubation. Therefore, development of effective
decellularisation protocols requires consideration of a number of variables to achieve an
acceptable balance between the removal of cellular components and preservation of
matrix proteins. Furthermore, as reviewed in Section 1.2.3, the combination of tissue
decellularisation and proteomic analyses can enhance the characterisation of the matrix
in any particular tissue.

1.4 Re-cellularisation of decellularised scaffolds

The ECM provides important cues to regulate cell behaviour (80, 237). Recellularisation of acellular scaffolds provide a model in which to study cell-matrix
interactions as well as the foundation for organ regeneration. A number of different
recellularisation strategies have been adopted for tissues or organs with different levels
of complexity.

1.4.1 Re-cellularisation of ECM from cultured cell sheets

Tondreau and colleagues used primary human dermal fibroblasts to create
artificial small-diameter vessel grafts for potential clinical use (238). The fibroblasts were
grown in plates (500cm²) and treated with ascorbic acid to stimulate ECM production and
generate sheets of fibroblasts on ECM. The sheets were then rolled around a rod to
create a blood vessel-like structure (collagen-rich, hollow tube) (238). This structure was then decellularised by submerging in water. Mechanical testing before and after decellularisation showed that decellularised “vessels” were able to resist the standard burst pressure used for testing of synthetic vascular prostheses (the ANSI/AAMI/ISO 7198:1998/2001 standard) suggesting that decellularisation process did not significantly damage the scaffold. The scaffold was then cannulated in a bioreactor, and endothelial cells (isolated from umbilical veins of patients) were infused into the lumen of the scaffold and incubated for 7 days with rotation. Histology showed a single layer of endothelial cells attached and repopulated the surface of the matrix, and mechanical tests demonstrated that the artificial vessel could also resist the burst pressure suggesting a possible use in implantation (238). In another study, ECM sheets produced by human periodontal ligament cells were decellularised using 20mM NH$_4$OH, 0.5% Triton X-100 and 100U/mL DNase I, to create a scaffold, with well-preserved structure and composition. SEM and confocal microscopy demonstrated that allogenic human periodontal ligament cells could attach to the scaffold, proliferate and deposit matrix throughout the culture period (21 days) indicating that this ECM sheet could support cell attachment and proliferation, and could be used in periodontal ligament tissue engineering in future (239). These studies demonstrate that recellularisation of scaffolds generated from cultured cells can provide tools for creating simple tissues.

1.4.2 Re-cellularisation of ECM from simple tissue structures

Scaffolds generated from tissues such as veins, adipose tissue and small intestine, which are more complex than those produced by cultured cell sheets can also be repopulated. For example, decellularisation of human greater saphenous veins with 0.075% SDS, produced scaffolds with preservation of structure and composition (240). Scaffolds were then implanted into the dog carotid artery, after 2 weeks endothelial cells from the circulation were observed to have attached to the scaffold creating tight vessels which were mechanically strong enough to sustain blood pressure (240). Repopulation of decellularised matrices has also been used in soft tissue reconstruction (241). Human
adipose tissue was decellularised using a combination of freeze-thaw, 0.25% trypsin/EDTA and 1% Triton X-100. Seven days after human adipose-derived stem cells (hASCs) (isolated from human adipose tissue) were seeded onto the matrix, cell attachment and proliferation were confirmed by IFL suggesting that this matrix provides a supportive environment for hASCs and potential a platform for generating new tissue (241). When acellular human adipose matrix scaffolds were implanted subcutaneously into rats (Fischer 344), H&E staining (241) indicated that the decellularised human tissue scaffold did not trigger an immune reaction and could support the infiltration of host cells and formation of blood vessels (241). Decellularised adipose matrix was also ground into a fine powder, mixed with hASCs and injected subcutaneously into the back of male nude rats, histology and IHC (using human-specific antibody) indicated that hASCs contributed to the formation of adipose tissue (241). These results suggested that adipose matrix could trigger angiogenesis and support blood vessel formation promoting cell infiltration and also promote differentiation of stem cells into adipose tissue which would have potential use in cosmetic surgery. It may also be possible to use matrices from one organ as the basis for the regeneration of a different organ, for example, the potential of using decellularised pig small intestine was tested for creating an oesophagus (197). The small intestine was decellularised by perfusion with 1% SDS and 1% Triton, and the matrix produced supported the growth of primary human oesophageal smooth muscle cells (197) which suggests that cells from a different organ could grow on the decellularised gut matrix. Taken together, the above studies show that decellularised matrix from simple-structured tissue can support the growth and differentiation of a variety of cell types which illustrate the potential for tissue regeneration.

1.4.3 Re-cellularisation of ECM from complex large organs

The ultimate goal of repopulating scaffolds is to regenerate more complex organs which contain unique structural features and multiple cell types (194). Rat heart was decellularised with a mixture of 1% SDS and 1% Triton X-100 and a mixture of neonatal rat cardiomyocytes, fibrocytes, endothelial cells and smooth muscle cells was injected
(multiple times) into either decellularised heart slices or whole hearts, and incubated either in a culture dish (slices; static culture) or in a bioreactor (whole heart; perfusion) for more than 10 days (192). Thicker cardiac muscle tissue (diameter: 250-1100µm) was generated in the whole heart scaffold compared to the slices (diameter: <50µm) and histological analysis and IFL of the recellularised whole rat heart scaffold showed that, at day 8 after seeding, cells could repopulate 1-2mm from the injection site with more the 95% cell viability suggesting that there might be a limited capacity for seeded cells to migrate away from injection site. However, the incubation period is relatively short, and it is possible that better repopulation would be achieved with an extended culture period. The expression of endothelial marker (von Willebrand factor (vWF)) was observed in some cells (data from recellularised heart slices were not reported) (192). The recellularised whole heart scaffold could also tolerate electrical stimulation up to 5Hz, which is similar to the refractory period of the adult rat heart while in the recellularised slices the maximum stimulation was 1Hz. This study showed that the decellularised heart scaffold could be repopulated by injection and perfusion with multiple cell types and support their growth, suggesting that decellularised heart scaffolds provide an illustration of the potential capacity of decellularisation technique to generate a platform for creating more complex multicellular organs. Whole livers from multiple species (pigs, rabbits, ferrets, rats and mice) have been decellularised with 1% Triton X-100 (242). The decellularised ferret liver scaffolds were repopulated by perfusion with GFP-labelled MS1 (MILE SVEN 1) endothelial cells (isolated from the endothelium of mouse pancreas and transformed by SV40) or a mixture of human umbilical vein endothelial cells (HUVECs) and human foetal liver cells (242). IFL demonstrated that MS1 endothelial cells repopulated the entire vascular system from larger vessels to capillaries. Data from the seeded cell mixture showed that these cells also repopulated the vascular system (cells expressed vWF, and the investigators suggested that these are mainly HUVECs) and expressed biliary epithelial cell marker in biliary tubular structures in the parenchyma. In addition, both hepatic and biliary epithelial cell marker was detected in some cells.
suggesting an immature hepatoblast phenotype. These results suggest that HUVECs attached to vascular matrices and proliferate and foetal liver cell proliferation and differentiation was induced by different matrices within the scaffold (242). Decellularised scaffolds can also be used to investigate how cells respond to different matrices. For example, whole human normal and fibrotic lung (IPF) was decellularised using 0.1% Triton X-100, 2% SDC and 30μg/ml DNase, cut into slices (1mm thick x 12mm diameter), and reseeded with primary normal human lung fibroblasts (158). IHC showed that fibroblasts grew on both matrices with cells seeded on the fibrotic matrix expressing significantly more cytoplasmic α-SMA, suggesting that the fibrotic matrix drove fibroblast to myofibroblast differentiation. In summary, these results indicate that decellularised matrix from complex organs can support the growth of multiple cell types and that individual cell types behave differently when seeded onto different matrices suggesting that these matrices can be used to study how ECM regulate cell behaviour.

1.4.4 Re-cellularisation of decellularised kidney ECM

The kidney is a complex, highly specialised organ containing more than 1 million nephrons (243). It contains more than 26 different cell types and possesses distinct matrix structures in different compartments (244). Within the kidney cortex, there are a number of different cell types including glomerular mesangial cells, podocytes and endothelial cells, proximal tubular epithelial cells, distal tubular epithelial cells, interstitial fibroblasts and vascular and capillary cells (243). As reviewed in Section 1.3.2.3, kidney tissues from a variety of species have been decellularised to create ECM scaffolds (244). The main focus of studies using these scaffolds has been on applications in tissue engineering with the ultimate aim of creating a transplantable kidney substitute (245). To achieve this, the decellularised scaffold needs to be repopulated with cells, given the multiplicity of cell types in the kidney, the selection of cell types and methods for efficient repopulation are of critical importance (245).

1.4.4.1 Re-cellularisation of decellularised kidney scaffolds using different cell types
As epithelial cells play an essential role in kidney function (246), this cell type is frequently used to recellularise kidney scaffolds. Immortalised human renal cortical tubular epithelial cells (RCTE cell line) were perfused into whole rat kidney scaffolds via the renal artery in a bioreactor (196). This study reported that high pressure could facilitate passage of epithelial cells through the vessel walls and the formation of tubular structures, however, the recellularisation was still incomplete (196). Song et al. used rat neonatal kidney cells (a mixture of different mainly epithelial cell types) together with HUVECs to repopulate a rat kidney scaffold by perfusion through the ureter and artery in combination with a pressure gradient (219). After 4 days of culture in a bioreactor, the vascular system, some tubules and all glomeruli were repopulated with cells. Interestingly, marker studies showed that epithelial (expressing aquaporin 1 (AQP1) and E-cadherin) and endothelial (expressing CD31) cells engrafted into their specific compartments of the kidney (219). In another study, Abolbashari et al. perfused primary pig renal tubular epithelial cells into a whole pig kidney scaffold (230). After 7 days in a bioreactor, tubule-like structures appeared (with expression of tubular markers (aquaporin 1 and 2 (AQP2))) which resembled those of the native kidney (230). However, the viability of seeded cells peaked at around 21 days of culture and significantly decreased at 28 days (230). The investigators did not propose any explanation for this observation, but it may be speculated that it may be due to an inadequate nutrient supply or potentially, the seeded cells reached the limit of their proliferative capacity since they were primary cells. Taken together, these results demonstrated that decellularised whole kidney matrix reseeded by perfusion, could support the attachment and growth of multiple cell types with some compartmentalisation of the cells and expression of differentiation markers.

The vascular system in the kidney not only supplies nutrients and oxygen but also plays a critical role in blood filtration (245). When engineering a kidney for transplantation, an intact vascular system is essential, thus, endothelial cells are frequently tested on/in kidney scaffolds (244). In several studies, whole porcine kidney
scaffolds were perfused with the MS1 endothelial cell line via the renal artery or vein (221, 229, 231), and cells were seen to re-endothelialise the renal artery, vein and cortical blood vessels (221, 229, 231) suggesting that this could form a platform for a clinically applicable bioengineered kidney. Song et al. also generated a re-endothelialised vascular system using HUVECs in rat kidney matrix scaffolds (219). In addition to endothelial cell lines, endothelial cells derived from human iPSC have been used to repopulate human and rat kidney ECM scaffolds and were shown to re-endothelialise glomeruli (162, 196). After re-endothelialisation, the various repopulated scaffolds were either perfused with blood or transplanted into rats or pigs and showed no sign of leaking (162, 196, 219, 221, 229, 231). Interestingly, these studies also demonstrated that there was significantly less platelet adhesion to the re-endothelialised vasculature compared with the un-seeded scaffold resulting in better patency of vasculature (221, 229, 231). In addition to studies on whole decellularised kidneys, human kidney peritubular microvascular endothelial cells (HKMECs) and HUVECs have been seeded onto gels derived from acellular human kidney ECM in which an artificial microvessel-like 3D structure had been created, and both endothelial cell types were shown to repopulate the micro-vessel structures suggesting that a 3D kidney ECM gel can also support endothelial cell growth with expression of endothelial markers such as CD31 and vWF (168). Taken together these studies show that decellularised kidney ECM (scaffolds or gels) can support growth and differentiation of endothelial cells creating vascular networks capable of supporting blood perfusion.

A mixed population of primary renal cells can be obtained by enzymatic digestion of kidney tissue and can be used to explore how multiple cell types behave when perfused into kidney scaffolds (244). He and colleagues used unselected cells digested from rat kidney tissue to perfuse rat kidney scaffolds and showed that the cells repopulated throughout the matrix and expressed a range of different cell type markers (proximal and distal epithelial, endothelial and podocyte) in different compartments of the kidney (216). As indicated above, primary rat and pig kidney cells (a mixed cell
population of predominantly epithelial cell types) were perfused into decellularised whole rat and pig kidney scaffold, respectively (219, 230). Both studies demonstrated that primary cells seeded into decellularised kidney ECM scaffolds form tubule-like structures which expressed different epithelial cell markers (AQP1 or AQP2) (219, 230).

Stem cells possess the potential to proliferate indefinitely and can differentiate into a variety of renal cell types (244, 247). Embryonic stem cells (ESCs), and in particular mouse ESCs (mESCs), have been used to repopulate decellularised kidney scaffolds (244, 245). A number of studies have infused decellularised rat kidney scaffolds with mESCs (193, 218, 225-227) and shown that the cells repopulate glomeruli, tubular structures and vascular structures with expression of the glomerular and tubular epithelial precursor marker (Pax-2), distal tubular epithelial marker (Ksp-cadherin), endothelial markers (CD31 and Tie-2) as well as an embryonic “stemness” marker (Oct-4) (193, 218, 225-227). Although the data on ESC repopulation of ECM scaffolds is promising, ethical concerns and the potential for teratomas might restrict the wider application of these cells (244, 248). iPSCs generated from adult somatic cells which retain the potential to differentiate into multiple renal cell types might avoid some of these concerns (249). As mentioned above, human iPSCs differentiated into endothelial cells and seeded into the whole rat and human kidney scaffolds successfully repopulated the endothelium (162, 196, 224). Human iPSCs have also been differentiated into renal progenitor cells (expressing Pax-2) and perfused into a mouse kidney scaffold via the renal artery with/without iPSC-derived endothelial cells (224). The renal progenitor cells reached the glomerular compartment and expressed the podocyte-specific marker, podocin. The cells also repopulated tubule-like structures and expressed both proximal (gamma-glutamyl transpeptidase (GGT) and sodium-glucose linked transporter 2 (SGLT2)) and distal (AQP2) tubular cell-specific markers, however, these progenitor cells appeared to be unable to differentiate into endothelial cells (224). These results suggested that the ECM can provide cues for iPSC-derived renal progenitor cells to differentiate into a variety of different renal cell types but not all cell types are represented.
1.4.4.2 Methods for repopulating decellularised kidney scaffolds

Perfusion is the most commonly used method for reseeding whole organ scaffolds as decellularisation of the organ preserves an intact vascular system. The renal artery and vein are frequently used to introduce cells into the scaffold, and perfusion by this route generally achieves 30-60% repopulation of the kidney (162, 193, 196, 216, 218, 219, 221, 224, 225, 227, 231). Cells have also been introduced via the ureter in combination with the renal artery and/or vein (219, 225, 227). For example, application of high pressure to the renal artery and ureter with negative pressure outside the scaffold was used to introduce a mixture of endothelial (artery) and epithelial (ureter) cells into the scaffold and generated glomerular, tubular and vascular-like structures within the matrix (219). Clearly, alternative methods of repopulation are required where there is no intact vasculature. Static culture (without perfusion and pressure) of cells seeded on top of kidney ECM cubes/slices has been used for repopulating decellularised ECM cubes or slices (245) (reviewed in Chapter 5, Section 5.1), however, this generally results in limited repopulation of the scaffold and optimisation of these systems is still required. Moreover, limited repopulation is also observed in studies of repopulating complex organs such as liver cubes (250), whole liver (242), whole heart (192) and whole pancreas (251). Although there have been advances in the repopulation of complex organs, this still remains a field which requires more technical advances.

1.4.4.3 Characterisation and assessment of repopulated kidney scaffolds

After the decellularised matrix has been seeded with cells, it is essential to characterise how cells localise and behave. Characterisation generally covers several aspects including ECM structure, cell adhesion, migration, proliferation and differentiation. Histological analysis (H&E staining) is frequently used to evaluate cell attachment and distribution within the recellularised scaffold (162, 193, 196, 211, 216, 218-222, 224-227, 231, 232, 235), and it can provide limited clues for cell-matrix interaction (244). Picro-Sirius red (PSR) staining is also used to analyse the changes in ECM structure and deposition of the collagens by seeded cells (220). Fluorescent
labelling of seeded cells, eg. GFP-labelled human renal cortical tubular epithelial cells, MS1 cells, and murine ESCs, also provides an effective means of monitoring cell distribution in the scaffold (193, 220, 221, 226, 229, 231). DAPI staining is frequently used to delineate cell nuclei in the scaffold (193, 196, 211, 226, 227, 231). As well as cell attachment and distribution within repopulated scaffolds, it is also important to evaluate aspects of cell function eg. proliferation and apoptosis (244, 245). Immunostaining for Ki67 and PCNA are commonly used to evaluate cell proliferation (162, 218, 226, 230, 235), while cleaved caspase-3 staining and TUNEL assays are used to assess apoptosis (230). The effect of the scaffold on differentiation of repopulating cells is also of interest. Studies have used a wide variety of cell type-specific differentiation markers which are generally assessed by immunostaining (Table 1.2).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Marker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podocytes</td>
<td>Podocin, nephrin, Wilms tumor-1 (WT-1), synaptotodin</td>
<td>(219)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Tie-2, CD31, VE-cadherin, Vascular endothelial growth factor (VEGF)-receptor 2, vWF</td>
<td>(162, 168, 216, 218, 219, 222, 224)</td>
</tr>
<tr>
<td>Embryonic stem cells</td>
<td>Oct-4</td>
<td>(218)</td>
</tr>
<tr>
<td>Proximal tubular epithelial cells</td>
<td>AQP1, Ezrin, γ-glutamyl transpeptidase (GGT), SGLT2</td>
<td>(216, 219, 224, 230)</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>E-cadherin</td>
<td>(219)</td>
</tr>
<tr>
<td>Distal tubule and collecting duct epithelia</td>
<td>AQP2, AQP4, Ksp-cadherin</td>
<td>(216, 219, 222, 224, 230)</td>
</tr>
<tr>
<td>Cell-cell adhesion</td>
<td>Ksp-cadherin</td>
<td>(222, 226)</td>
</tr>
<tr>
<td>Renal progenitor cells (also a glomerular epithelial cell marker)</td>
<td>PAX-2</td>
<td>(222, 226)</td>
</tr>
<tr>
<td>Mesenchymal cell marker</td>
<td>Vimentin</td>
<td>(232)</td>
</tr>
</tbody>
</table>

Table 1.2 Markers used in published studies of the recellularization of the kidney matrix. AQP1: aquaporin 1; AQP4: aquaporin 4; SGLT2: sodium-glucose linked transporter 2;

In summary, examples from simple cell sheets to complex organs show that decellularised ECM scaffolds can support the attachment and growth of reseeded cells and may provide cues for cell type-specific differentiation which make recellularisation of
matrix scaffolds both a promising platform for the tissue regeneration and a tool to study how the ECM interacts with specific cell types and regulates cell phenotype and function.

1.5 Aims

The overall goal of this thesis was to analyse the human kidney cortical ECM and investigate how cell-ECM interactions in the human kidney cortex regulate cell growth and differentiation. The study addressed three specific aims to:

1. Characterise the ECM of the human kidney tubulointerstitium using an ECM enrichment technique in combination with proteomics, and to compare the tubulointerstitial and glomerular ECM and examine age-related changes in these matrices.

2. Based on the protocols developed for pig kidney tissue cubes to develop a protocol for decellularising human kidney cortex tissue cubes and compare ultrastructure and protein profile of the matrix scaffold with the ECM of intact tissue.

3. Then examine the effect of decellularised human kidney cortex scaffolds on the growth and differentiation of selected human kidney cortical cell types.
Chapter 2

Materials and Methods
The methods described in this chapter are those used in more than one chapters, specific methods will be described in the methods section of each chapter.

2.1 Chemicals

All chemicals were obtained from Sigma Aldrich (Gillingham, UK) unless otherwise stated.

2.2 Human kidneys

Human kidneys anatomically unsuitable for transplantation, were collected under ethical approval (Ethics Number 05/Q0508/6 (UCL), and G12-42012A (UCL) and 06/Q1406/38 (University of Manchester)). In general, kidneys were perfused at collection and shipped in perfusion solution on ice. Under sterile conditions kidneys were decapsulated, cut into transverse slices ~5-10mm in thickness, the slices placed in individual Whirlpak bags, snap frozen or slowly frozen to -80°C. All tissue samples were then stored at -80°C for experiments. Tissue samples were fixed in 4% paraformaldehyde and stained with H&E (Section 2.4). Histology of each kidney was independently accessed by a renal pathologist (Dr Paul Bass, Department of Cellular Pathology, Royal Free NHS Foundation Trust), the details of the kidneys used in this study are shown in Table 2.1.

2.3 Cell culture

All cells were obtained from archived cryogenic stocks stored in liquid nitrogen. All cell culture plasticware was from Corning (High Wycombe, UK).

Human proximal tubular epithelial cells (HK2) were plated in T75 flasks (Corning) in Dulbecco's modified Eagle's Medium (DMEM; Gibco: 31966-021; ThermoFisher, Rochford, UK) with 10% foetal bovine serum (FBS; Sera Laboratories International, Haywards Heath, UK) and 100U/ml penicillin, 100µg/ml streptomycin and 250ng/ml amphotericin B (antibiotic-antimycotic solution; ThermoFisher) and cultured at 37°C in
<table>
<thead>
<tr>
<th>Kidney ID</th>
<th>Age</th>
<th>Gender</th>
<th>Histology</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHK15</td>
<td>15</td>
<td>Male</td>
<td>Normal; good tissue architecture</td>
<td>Proteomics</td>
</tr>
<tr>
<td>NHK29</td>
<td>29</td>
<td>Male</td>
<td>Generally normal with very occasional interstitial fibrotic foci.</td>
<td>Proteomics and Decellularisation</td>
</tr>
<tr>
<td>NHK40</td>
<td>40</td>
<td>Male</td>
<td>Generally normal with well perfused glomeruli and patent tubules. Some thickening of large vessel walls. Occasional areas of inflammation.</td>
<td>Decellularisation</td>
</tr>
<tr>
<td>NHK60</td>
<td>60</td>
<td>Female</td>
<td>Changes consistent with age. Occasional ischemic/sclerosed glomeruli and atrophic tubules containing proteinaceous casts. Evidence of benign degenerative vascular disease with duplication of the elastic laminae. Occasional fibrotic tracts in the interstitium.</td>
<td>Decellularisation</td>
</tr>
<tr>
<td>NHK61</td>
<td>61</td>
<td>Male</td>
<td>Changes consistent with age. Glomeruli generally well perfused. Some atrophic tubules with occasional calcified deposits. Patches of inflammation and fibrotic foci in the interstitium.</td>
<td>Proteomics, Decellularisation and Recellularisation</td>
</tr>
<tr>
<td>NHK64</td>
<td>64</td>
<td>Female</td>
<td>Changes consistent with age. Some sclerosed glomeruli. Occasional calcified tubules. Moderate benign vascular changes with thickening of the vessel walls and duplication of the elastic laminae. Some areas of inflammatory cell infiltrate.</td>
<td>Decellularisation and Recellularisation</td>
</tr>
<tr>
<td>NHK67</td>
<td>67</td>
<td>Not known</td>
<td>Changes consistent with age. Glomeruli generally well perfused. Some tubules with irregular profiles possibly indicative of acute (ante-mortem) tubular damage. Occasional foci of inflammatory cells.</td>
<td>Proteomics and Decellularisation</td>
</tr>
<tr>
<td>NHK68</td>
<td>68</td>
<td>Female</td>
<td>Changes consistent with age. Generally good preservation of tissue architecture. Fibroproliferative changes in the vasculature. One “nest” of densely-staining epithelioid cells, possibly an early tumour.</td>
<td>Decellularisation</td>
</tr>
<tr>
<td>NHK69</td>
<td>69</td>
<td>Male</td>
<td>Changes consistent with age. Occasional sclerosed glomeruli. Tubules generally well preserved. Some thickening of vessel walls. Occasional fibrotic foci.</td>
<td>Decellularisation and Recellularisation</td>
</tr>
<tr>
<td>NHKNK</td>
<td>Not known</td>
<td>Not known</td>
<td>Normal; Occasional calcified tubules (unclear if calcification is luminal or epithelial cells have calcified). Some calcification also present in the deeper medulla.</td>
<td>Decellularisation</td>
</tr>
</tbody>
</table>

**Table 2.1** Age, gender and histology of human kidneys used in the study.
a humidified atmosphere of 5% carbon dioxide (CO\textsubscript{2}). Medium was changed every 2 days. At confluence, cells were passaged using 0.05% Trypsin-EDTA (ThermoFisher), neutralised using complete medium with 10% FBS (volume is 4 times greater than volume used for trypsinisation) and split 1:4 or 1:8 into new flasks (T75) depending on the time scale for the experiments.

Conditionally-immortalised human kidney fibroblasts (252) were plated in T150 flasks and cultured in DMEM containing 10% FBS and antibiotic-antimycotic solution in a humidified incubator with 5% CO\textsubscript{2} at 33°C (permissive temperature). At confluence cells were passaged at a 1:2 - 1:4 split. For experiments cells were passaged 1:2 into new T150 flasks, grown overnight at 33°C and then transferred to 37°C (non-permissive temperature) and grown for 2 weeks. Medium was changed every 3-4 days at both temperatures.

Primary human normal kidney fibroblasts and primary human skin fibroblasts were plated in T150 flasks in Dulbecco’s modified Eagle’s Medium (DMEM) with 10% FBS and antibiotic-antimycotic solution and cultured at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. Medium was changed every 2 days. At confluence, the cells were passaged using Trypsin-EDTA (ThermoFisher) and split 1:2 (primary human normal kidney fibroblasts) and 1:4 (primary human skin fibroblasts) into new T150 flasks.

Conditionally-immortalised podocytes (253) were plated in T150 flasks and cultured in RPMI 1640 medium (Gibco: 31870-025; ThermoFisher) with 10% FBS, 1mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin (ThermoFisher), 10mg/L insulin, 5.5mg/L transferrin, and 6.7µg/L selenite (ITS, ThermoFisher) in a humidified incubator with 5% CO\textsubscript{2} at 33°C. At confluence, cells were passaged using 0.05% Trypsin-EDTA (ThermoFisher) and split 1:4 - 1:8 into new T150 flasks. To differentiate the conditionally-immortalised podocytes, at passage cells were split 1:2 - 1:4, grown overnight at 33°C and then transferred to 37°C for 2 weeks in RPMI 1640 medium with 10% FBS, 1mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Medium was changed every 3-4 days at both temperatures.
For freezing cells, the following procedure was applied to all cell types. When cells reached 70 – 80% confluence, the cells were washed twice with phosphate-buffered saline (PBS; ThermoFisher). Cells were then trypsinised to form a single cell suspension, trypsin activity neutralised with complete medium (volume is 4 times of the volume used for trypsinisation), transferred to a 15 ml Falcon tube and centrifuged at 200xg for 5 minutes at 37°C. Cell pellet was gently re-suspended in FBS containing 10% DMSO (freezing medium), aliquoted in 1ml volumes in 1.5ml cryovials (ThermoFisher), placed in a Mr Frosty overnight at -80°C and then transferred to liquid nitrogen for long-term storage.

### 2.4 Immunohistochemistry (IHC) and immunofluorescence (IFL)

Tissue samples were fixed with 4% paraformaldehyde (TAAB Laboratories, Sandhurst, UK) (volumes greater than 10 times of tissue volume) overnight at 4°C on a roller followed by 2 x 10-minute washes with PBS (+MgCl₂ and CaCl₂; all PBS used throughout this section contains MgCl₂ and CaCl₂) and 1 x 2 hours wash at room temperature. Fixed samples were then left in 4°C overnight. Samples were dehydrated through graded alcohols to xylene, embedded in paraffin wax and sectioned (5µm) (C&C Laboratory Services, London, UK). Paraffin-embedded tissue sections (5µm) were rehydrated and washed twice in PBS/0.1% Tween-20.

For H&E staining, after rehydration, sections were washed with water and stained with haematoxylin for 5 minutes followed by rinsing with water twice for 5 minutes each. Sections were stained with eosin for 2 minutes and washed with water for 3 minutes. Stained sections were then dehydrated through graded ethanol (70 – 100%, v/v), washed twice with xylene and mounted (Mounting medium; Dako, Stockport, UK) with a coverslip for microscopy examination.

For IHC, sections were boiled in a pressure cooker in citrate buffer (0.01M, pH6) for 25 minutes and cooled to room temperature. PAP Pen (ImmEdge; VWR, Lutterworth, UK) was used to create a hydrophobic barrier around the section to secure the primary
antibody. Sections were then washed in PBS with 0.1% Tween 3 times for 5 minutes each and blocked in 10% (v/v) goat serum, 1% (w/v) bovine serum albumin (BSA) in PBS for 1 hour at room temperature followed by avidin blocking for 15 minutes and biotin block for 15 minutes (Vector Laboratories, Peterborough, UK). Primary antibodies (Table 2.2) were diluted in blocking solution. Sections were incubated with primary antibody at 4°C overnight in a humidified chamber then washed with PBS/0.1% Tween 3 times for 5 minutes each. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxide solution for 15 minutes. Secondary antibodies (biotinylated anti-rabbit antibody (Vector Laboratories, BA-1000) and biotinylated anti-goat antibody (Vector Laboratories, BA-9500)) were diluted in blocking solution and sections incubated for 1 hour at room temperature, followed by avidin-biotin peroxidase incubation for 45 minutes at room temperature (Vectastain ABC; Vector Laboratories). Antibody binding was visualised using the chromogenic substrate, 3-amino-9-ethylcarbazole (AEC; Vector Laboratories), for 10-30 minutes at room temperature. Sections were counterstained with haematoxylin (Dako) for 1 minute followed 2 x 3 minute-water washes. Finally, the sections were mounted with aqueous mounting medium (VectaMount; Vector Laboratories).

For IFL, after rehydration, sections were boiled in pressure cooker in citrate buffer (0.01M, pH6) for 25 minutes and cooled to room temperature. PAP Pen (ImmEdge) was used to create hydrophobic barrier to secure the primary antibody. Sections were then washed in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS/T) 3 times for 5 minutes each. Sections were then blocked in 10% (v/v) goat serum, 1% (w/v) BSA in TBS with 0.1% Tween (TBS/T) for 1 hour at room temperature. Primary antibodies (Table 2.2) were diluted in blocking solution. Sections were incubated with primary antibody at 4°C overnight in humidified chambers and washed with TBS/T 3 times for 5 minutes each. Secondary antibodies (anti-mouse Alexa Fluor 568, anti-rabbit Alexa Fluor 568) were diluted in blocking solution and sections incubated for 1 hour at room temperature. Sections were then rinsed with TBS/T 3 times for 5 minutes each. DAPI (diluted 1:100 in PBS; ThermoFisher) was applied for 5 minutes, sections washed with TBS/T (twice, 5
minutes each) and mounted with Fluoroshield anti-fade mounting medium (Millipore, Watford, UK). Images were captured using an Axioskop 2 mot plus microscope (Zeiss, Cambridge, UK) and the multispectral imaging system (AxioCam MRm, Zeiss). At least 10 fields/section were examined, and representative images were selected.

IFL for Fibulin-1 was performed with Dr Maria Kolatsi, UCL Institute of Child Health. After rehydration, sections were boiled in citrate buffer (0.01M, pH6) for 15 minutes and cooled to room temperature. PAP Pen (ImmEdge) was used to create a hydrophobic barrier to secure the primary antibody. Sections were then washed in TBS/T 3 times for 5 minutes each. Sections were blocked in 10% (v/v) FBS, 1% (w/v) BSA in PBS/0.1% Tween for 1 hour at room temperature. Primary antibodies were diluted in blocking solution with 10% goat serum. Sections were incubated with primary antibody at 4°C overnight in humidified chamber and washed with PBS/T 3 times for 5 minutes each. Secondary antibodies (anti-mouse Alexa Fluor 568 (ThermoFisher, A-11004, 1:300)) were diluted in blocking solution with 10% (v/v) goat serum and sections incubated for 1 hour at room temperature. Sections were then rinsed with PBS 5 times for 5 minutes each. Hoechst 33342 (ThermoFisher; 1:100 diluted in PBS) was applied for 5 minutes, and sections washed with PBS 3 times for 5 minutes each and mounted with SlowFade mounting medium (ThermoFisher). Images were captured using an Axiophot microscope (Zeiss) and the multispectral imaging system (AxioCam MRc5, Zeiss). Ten fields/section were examined, and representative images were selected.

2.5 Proteomics of human kidney ECM

2.5.1 ECM enrichment

Samples from Section 3.2.1 and Section 4.2.1 were stored at -20°C. Samples were defrosted at room temperature, homogenized using a 21G needle and 2ml syringe (BD Biosciences) and centrifuged at 14,000 x g for 5 minutes at room temperature and the supernatant removed (139). Ice-cold TB solution (500μl; 10mM Tris, 150mM sodium chloride, 25mM EDTA, 1% (v/v) Triton X-100, 25μg/ml leupeptin, 25μg/ml aprotinin,
0.5mM 4-(2-aminoethyl)benzenesulfonfyl fluoride hydrochloride (AEBSF)) was added to the pellet and samples incubated on ice for 1 hour. Samples were centrifuged at 14,000 x g for 10 minutes at 4°C, the supernatant was collected and mixed with 125μl recovery buffer (15 % (w/v) SDS, 100mM 1,4-dithiothreitol (DTT), 200mM Tris, 30% (v/v) glycerol, 0.01% Bromophenol blue). This sample was designated the “cellular fraction” (most of

<table>
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<th>Catalogue number</th>
<th>Dilution</th>
</tr>
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<tr>
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<td>Santa Cruz</td>
<td>sc-25281</td>
<td>1:200</td>
</tr>
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<td>Abcam</td>
<td>Ab9566</td>
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Table 2.2 Antibodies used in IHC and IFL.

![Figure 2.1 Proteomics workflow.](image-url)
the cellular components separate into this cellular fraction) and stored at -80°C. The pellet was re-suspended in 500μl ice-cold EB solution (20mM NH₄OH, 0.5% (v/v) Triton X-100) and incubated on ice for 1 hour. Samples were centrifuged at 14,000 x g for 10 minutes at 4°C, the supernatant collected, designated the “soluble fraction 2” and stored at -80 °C. The pellet was re-suspended in 500μl PBS with DNase I (25µg/ml) and incubated for 30 minutes at room temperature. Samples were centrifuged at 14,000 x g for 10 minutes at 4°C, the supernatant collected, designated the “soluble fraction 3” and stored at -80°C. The final pellet was re-suspended in 200μl recovery buffer and incubated for 10 minutes at 95°C to give the ECM-enriched fraction. Samples were cooled to room temperature and stored at 4°C (Figure 2.1).

2.5.2 Mass spectrometry (MS)

2.5.2.1 Preparation of protein samples

Aliquots (10μl) of soluble cellular fraction and the ECM-enriched fraction of each sample were run on 4-12% Bis-Tris Polyacrylamide gels (ThermoFisher) with a molecular weight ladder covering the molecular weight range 10 – 250 kDa (Precision Plus Protein; Bio-Rad, Watford, UK) in NuPAGE® MES SDS Running Buffer (diluted from 20x stock with dH₂O; ThermoFisher) at 200V until the bromophenol blue dye front reached the bottom of the gel. Gels were stained with Instinct Blue (Expedeon, Cambridge, UK) briefly and washed with distilled H₂O overnight. Total protein of each sample was quantified on an Odyssey CLx Infrared Imaging System (Li-Cor Biosciences, Cambridge, UK) and normalized to the lowest sample concentration.

Samples were then run on a second 4-12% Bis-Tris Polyacrylamide Gel until the samples were about 10mm into the gel (~ 5 minutes). The gel was stained with Instinct Blue (Expedeon) at 4°C overnight, and then de-stained with 5 washes of 5 minutes in MilliQ water (MQ-H₂O; 18.2MOhm; Millipore). The 96-well plate assembly was assembled with the perforated plate (ThermoFisher) on top of the gel and U-shaped bottom storage plate underneath. The lanes were then excised from the gel using clean blades and each lane was sliced and transferred into individual wells of the top plate (96-
well) of the assembly (1 lane/well). MQ-H₂O was added into the well to prevent the gel from drying out and the plate assembly stored at 4°C overnight. The plate assembly was centrifuged at 210 x g to remove the water and transferred to the fume cupboard. The storage plate was emptied, and the gel slices washed repeatedly in 50μl 50% acetonitrile: 50% 25mM NH₄HCO₃ until completely transparent (plates were centrifuged at 210 x g for 1 minute after each wash). The gel slices were then washed with 50μl acetonitrile twice, 5 minutes each (centrifuged at 210 x g for 1 minute after each wash). The gel slices were dried without the collection plate, in a vacuum centrifuge (Savant SPD131DDA; ThermoFisher) for 30 minutes in a rotor for 96-well plate at room temperature. DTT solution (1.25mM DTT in 25mM NH₄HCO₃) was added (50μl/slice) for 1 hour at 56°C to denature the proteins in the gel slices. The plate assembly was centrifuged at 210 x g for 1 minute. Iodoacetamide solution (40mg Iodoacetamide in 5ml MQ-H₂O water) 50μl/sample, was added to the plate and incubated at room temperature in the dark for 45 minutes then centrifuged at 210 x g for 1 minute. The gel slices were washed with 50μl 25mM NH₄HCO₃ for 10 minutes followed by centrifugation at 210 x g for 1 minute. The slices were then washed with 50μl acetonitrile for 5 minutes followed by centrifugation at 210 x g for 1 minute. The last 2 wash steps were repeated, and the plate dried for 25 minutes in a vacuum centrifuge at room temperature. The collection plate was replaced, 5μl 10x trypsin (Promega) added to each well and incubated for 40 minutes at 4°C. After incubation, 45μl 25mM NH₄HCO₃ was added and the plate assembly incubated overnight at 37°C. The plate assembly was then centrifuged at 210 x g for 1 minute. Acetonitrile:formic acid (100%:0.2%) 50μl/sample was added and incubated for 30 minutes at room temperature, followed by centrifugation at 210 x g for 1 minute. Acetonitrile:formic acid (50%:0.1%) 50μl/sample was added for 30 minutes at room temperature, followed by centrifugation at 210 x g for 1 minute. The pooled supernatants (150μl) were then dried completely in a vacuum centrifuge. Peptides, in the 96-well plate, were re-suspended in 20μl acetonitrile:formic acid (5%:0.1%) and stored at -20°C.
2.5.2.2 MS detection

POROS R3 beads (10μl, 0.1mg/μl; Applied Biosciences, Beverly Hills, California, USA) were placed in each well of a new 96-well plate with a 0.2μm Polyvinylidene difluoride (PVDF) membrane (Corning) in each well. Wet solution (50% (v/v) acetonitrile) was added, 20μl/sample, and mixed by pipetting 5 times followed by centrifugation at 210 x g for 1 minute at room temperature. Wash solution (0.1% (v/v) formic acid) was added (20μl/sample) and mixed by pipetting 5 times followed by centrifugation at 210 x g for 1 minute.

The plate (with re-suspended peptides; described in Section 2.5.2.1) was removed from the -20°C freezer, the samples were added into individual wells of the 96-well plate prepared (as described above), and mixed 5 times followed by centrifugation at 210 x g for 1 minute (the flow-through was retained in case repeated analysis is required). Wash solution was added (20μl/sample) and mixed by pipetting 5 times followed by centrifugation at 210 x g for 1 minute at room temperature. Elution solution (20μl/sample; 50% acetonitrile:0.1% formic acid) was added and mixed 5 times followed by centrifugation at 210 x g for 1 minute (the flow-through was retained). Samples were then dried completely in a vacuum centrifuge and the peptides re-suspended in 20μl 50% acetonitrile:0.1% formic acid. The samples were then ready for LC-MS/MS detection (ThermoFisher). LC-MS/MS was performed by Dr. Michael Randles in the Biological Mass Spectrometry, Core Facility, Faculty of Life Sciences, University of Manchester.

Raw MS data were analysed and quantified by Progenesis QI (Nonlinear Dynamics, Newcastle upon Tyne, UK), and searched with Mascot (Matrix Science Ltd, London, UK) against the UniProt Human database (modified 28th August 2015, UniProt Consortium), allowing one missed cleavage, containing carboxamidomethylation (C) as a fixed modification and oxidation of methionine, lysine and proline as variable modifications. The peptide tolerance values were set as 5 ppm and error window for MS/MS fragment ion mass tolerance value was 0.5 Da. Peptide charge was set as 2+, 3+ and 4+. MS datasets were then implemented in Scaffold (version 4.40; Proteome
Software, Portland, Oregon, USA). The spectral count was derived from the identification of MS/MS spectra and normalised to molecular weight of each protein. The sequences of unique peptides used for identification of collagen isoforms were listed in Appendix Table 2.1.
Chapter 3

Composition of the human kidney
tubulointerstitial and glomerular matrisome in
health and ageing
3.1 Introduction

The ECM provides a critical support structure for cells and also initiates outside-in cell signal transduction (11). Individual ECM proteins may have multiple functions such as structural support and regulating cell behaviours, therefore, to understand the regulatory function of the ECM, it is important to gain insights into the detailed composition of the matrix (254). As reviewed in Chapter 1, Section 1.1.3, information on the composition of the kidney ECM has largely come from candidate-based experimental approaches, which have defined the importance of key ECM components such as collagen IV and laminin isoforms (9, 255, 256). The protein composition of the ECM has generally been analysed by immunostaining, and Western blotting (49, 138, 218). However, these techniques can only examine a relatively small number of selected proteins and are dependent on the availability of reagents for target detection. Therefore, an alternative non-biased, high capacity approach is required to study the complexity of tissue-specific ECM composition and changes in the ECM that occur in disease. Proteomic techniques provide such an approach and more recently proteomics coupled with ECM enrichment techniques (7) have been used and have revealed the complexity of the ECM within individual tissue compartments.

This study focussed on the cortex of human kidney as it accounts for majority of kidney volume. In terms of the human kidney cortical matrix to date proteomics have only been applied to study the glomerular ECM (139, 167). Differential sieving (139) or laser capture microdissection (167) were used to enrich matrix proteins identification. After ECM enrichment, 144 and 147 matrix proteins were identified by Lennon et al. and Hobeika et al. respectively, using MS-based proteomics. In addition to the glomerular matrix, the cortex contains the tubulointerstitial (TI) matrix comprised of tubular basement membranes, vascular matrices and the interstitial ECM. However, as reviewed in Chapter 1, Section 1.2.3.2.1, unlike the glomerular ECM, there is relatively little known about the composition of TI matrix with the majority of information coming from candidate-based studies. This is perhaps surprising, given that it is the changes in the
TI matrix (accumulation of ECM in this compartment) which provides the best prognostic indicator of progression to end-stage renal disease (ESRD) in CKD (184). Candidate-based studies have identified collagens (55), LAMs (8, 59), fibronectin (60), decorin (55) and biglycan (55) as components of the TI ECM in both healthy and diseased human kidneys. However, to date no studies have examined the human TI ECM using proteomic approaches.

In the kidney, ageing leads to significant structural and functional changes (136) including inflammation, the appearance of fibrosis and changes in vascular tone which are associated with declining renal function (136, 185, 186). Within the kidney, the decline of cortex volume has been shown to starts around 18 years of age which is much early than the decline of total kidney volume which becomes apparent around 50 years of age (257). The matrix also varies with ageing and information of how kidney ECM changes with age in glomerular and tubulointerstitial matrix are available from candidate-based approaches. IHC showed increased expression of collagen IV in glomeruli of aged mice (27 months) compared with young mice (3 months) (82). Using Sirius red staining and immunoblotting, both collagen I and III were elevated in the tubulointerstitium of 26-32 month C57Bl/6 mouse kidney compared with 4-6 month-old kidneys (83). Proteomics have been used to study changes in the ageing of kidney, however, most of these studies used whole kidney tissues from animal models. For example, using MS-based proteomics, 103 proteins were identified as significantly altered (2-fold increase/decrease in expression) with age when kidney tissues from 13 month-old male Fisher rats and from 31 month-old rats were compared (177). The available data highlight age-related changes in the human kidney ECM and suggest a more comprehensive analysis is warranted.

The work presented in this chapter used MS-based proteomics to address three specific aims: i) the characterisation of human cortical TI matrix; ii) a comparison of the composition of the TI matrix with the glomerular matrix from the same kidneys; and, iii) the identification of age-related changes in TI and glomerular matrices.
3.2 Material and Methods

3.2.1 Proteomics of human kidney cortex

3.2.1.1 Separation of glomeruli and tubulointerstitium

Six human kidneys (Young group: 15, 29 and 37 years of age; Aged group: 61, 67 and 69 years of age) (Table 2.1) were collected under ethical approval (see Chapter 2, Section 2.2) and tissue samples stored at -80°C. Kidney cortex was dissected from frozen human kidney slices (139). Tissue (2g) was placed in a glass petri dish and cut into small pieces (≤1mm³). Graded sieves 250µm, 150µm and 106µm (Endecotts, London, UK) were stacked with the 250µm sieve at the top and placed on a collection container. Tissue pieces were transferred onto the largest sieve in 5ml ice-cold PBS (without Ca²⁺ and Mg²⁺ (-Ca/Mg)). The plunger from a 10ml syringe was used to press the tissue through the sieves. Fragments were washed through the sieves with 60 – 100ml of ice-cold PBS. Glomeruli were retained on the 150µm and 106µm sieves and were collected by inverting the sieves and washing with 20ml ice-cold PBS. The cortical TI fragments were collected from the flow-through by centrifugation. The glomeruli and TI samples were centrifuged at 3,000 x g for 3 minutes at 4°C then washed 3 times by centrifugation and re-suspension in 10ml ice-cold PBS. After the final wash, samples were re-suspended in 5ml PBS and aliquoted into five 1.5ml microcentrifuge tubes. One aliquot from each sample was fixed in 4% PFA, processed, paraffin-embedded and stained with H&E to confirm the separation of glomeruli and TI tissue. One aliquot was frozen at -20°C for ECM enrichment and the remainder stored at -80°C for future use.

Proteomic analysis was performed as described in Chapter 2, Section 2.5. Briefly, samples were enriched for ECM using detergents, DNase and proteinase with centrifugation. Enriched samples were then run on 4-12% Bis-Tris Polyacrylamide gels for separation before de-saltation and prepared for MS detection.

3.2.1.2 Bioinformatic analyses of MS datasets

MS datasets were implemented in Scaffold (Proteome Software). Protein identifications were accepted upon assignment of at least 2 unique validated peptides
with ≥90% probability, resulting in ≥99% probability at the protein level. These acceptance criteria resulted in an estimated protein false discovery rate (FDR) of 0.1% for all datasets. Proteins were accepted as present in either the T1 or glomerular ECM proteome if they were present in at least 2 of 6 biological replicates with at least 2 spectral counts. The Human Matrisome Project (HMDB (2) (http://matrisomeproject.mit.edu) was used to identify known ECM proteins. ECM enrichment of each sample was calculated using the total spectral count of ECM proteins divided by the total number of spectral counts of all proteins identified by MS.

All identified proteins were analysed using DAVID gene ontology (The Database for Annotation, Visualization and Integrated Discovery, version 6.8). The GO biological process annotation chart (GOTERM_BP_FAT) was selected (FDR<0.01, p<0.005). The list of proteins was also imported into Cytoscape (version 3.3.0). Markov Cluster Algorithm (MCL) was used to analyse the protein list. Age-related differences were determined using Two-way ANOVA with Bonferroni correction between the young and aged groups in T1 and G ECM, and the p< 0.05 was considered significant. Hierarchical clustering was performed to determine the age-related differences using Euclidean distance correlation and complete linkage matrix in MultiExperiment Viewer (258).

Protein interaction network analysis was performed using Cytoscape (version 3.4.0) (259). Proteins identified in at least 2 biological replicates were mapped onto a merged human interactome built from the Protein Interaction Network Analysis platform Homo sapiens network (release date, 10 December 2012) and Mus musculus network (release date, 10 December 2012) (260), the ECM interaction database MatrixDB (release date, 20 April 2012) (261, 262), and a literature-curated database of integrin-based adhesion–associated proteins (263).

Official gene names and accession numbers were used to identify proteins in the Uni_Prot database. Protein function and organ-specificity information were exported from Uni_Prot. Official gene names were searched with keywords: “kidney extracellular matrix”, “kidney matrix”, “kidney”, “human kidney matrix”, “human kidney” and “human”
against PubMed to determine the reported tissue-specific expression of each identified protein. In order to be inclusive, results of RNA profiling, genetic studies and protein expression studies from the published papers were used to determine the identification and distribution of proteins in different tissues of different species. IHC data from published papers were also used to identify the localisation of proteins in glomeruli and/or the TI.

3.2.2 Western blotting

Aliquots (10μl) of the ECM fractions were run on denaturing sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using a 4% stacking gel and 10% resolving gel using the Mini PROTEAN® Tetra cell system (Bio-Rad). Gels were run in SDS-PAGE running buffer (192mM Glycine, 25mM Tris, 0.1% SDS) at 50V for 30 minutes and then increased to 100V until the dye-front reached the bottom of the gel (at this point gels could be also used for Coomassie Blue and Silver staining for total protein quantification (Section 3.2.3, 3.2.4)). The gel was then soaked in transfer buffer (25mM Tris, 190mM Glycine, 0.1% SDS, 20% Methanol) for 5 minutes. Proteins were transferred onto 0.2µm pore nitrocellulose membrane (GE Healthcare, Amersham, UK) using the Mini Trans-Blot® Cell system (Bio-Rad) at 350mA for 90 minutes. After transfer, gels were stained with Coomassie Blue staining and membranes were stained with Ponceau S to access transfer consistency and efficiency by visual examination. Membranes were de-stained in water and PBS then blocked in blocking solution (5% fat-free semi-skimmed milk (Marvel, Thame, UK) in TBS/T (50mM Tris, 150mM NaCl, 0.1% Tween, pH7.5)) for 1 hour at room temperature with shaking at 35 rpm (Rocking shaker, Stuart). Primary antibody was diluted in blocking solution and membranes incubated in diluted primary antibody solution overnight at 4°C. Membranes were washed with TBS/T 3 times for 10 minutes each. Secondary antibody (conjugated with horseradish peroxidase) was diluted in blocking solution and membranes incubated for 2 hours at room temperature with shaking at 35 rpm (Rocking shaker, Stuart, Stone, UK). Membranes were then washed with TBS/T 3 times for 10 minutes each followed by PBS rinsing (5 - 10 minutes) then
incubated with chemiluminescence substrate (LumiGLO®, Cell Signalling, Danvers, Massachusetts, USA) for 1 minute. The UVP Biospectrum 810 imaging system (UVP, Cambridge, UK) was used to image the blots.

3.2.3 Coomassie blue

After electrophoresis, some gels were stained with Coomassie Blue solution (3mM Coomassie Blue, 45% methanol, 10% acetic acid) for 30 minutes at room temperature with shaking at 35 rpm (Rocking shaker, Stuart). Gels were washed with de-stain buffer (45% methanol, 10% acetic acid) 3 times for 15 minutes each with shaking at 35 rpm (Rocking shaker, Stuart), imaged with UVP Biospectrum 810 imaging system and analysed with UVP software (UVP).

3.2.4 Silver staining

After electrophoresis, gels were fixed in 40% ethanol, 10% acetic acid for 35 minutes at room temperature. Fixed gels were washed with water overnight for 4°C with shaking at 35 rpm (Rocking shaker, Stuart). Gels were sensitised in 0.02% sodium thiosulfate for 1 minute followed by two 1 minute water washes. Gels were then incubated with ice-cold silver nitrate solution (0.1% silver nitrate, 0.007% formaldehyde) for 20 minutes followed by three 1 minute water washes. Gels were developed in sodium carbonate (3% sodium carbonate, 0.0175% formaldehyde). After developing, gels were washed in water for 20 seconds and incubated in 5% acetic acid for 5 minutes. Gels were stored in 1% acetic acid at 4°C. Stained gels were imaged with the UVP Biospectrum 810 imaging system and analysed with UVP software (UVP).

3.2.5 IFL and Image analysis

For details of IFL for Fibulin-1 on human kidney sections see Chapter 2, Section 2.4. Histology images of human kidney (H&E stained) were acquired using an Axiophot microscope (Zeiss) and the Trio multispectral imaging system (Caliper). IFL Images were captured using an Axiophot microscope (Zeiss) and the multispectral imaging system (AxioCam MRc5, Zeiss). IFL images were analysed using ImageJ (Version 1.51j8). Total positive staining was measured in glomeruli and TI separately, and then normalised to
the total background in the same area of staining to generate corrected total fluorescence. Twenty fields from a single section of each kidney were examined. The data were assumed to be normally distributed and presented as mean ± standard deviation (SD).

3.3 Results

3.3.1 Isolation and enrichment of TI and glomerular ECM from human kidney tissue

Kidney cortex was isolated from 6 donor human kidneys anatomically unsuitable for transplantation, ranging in age from 15-69 years. Glomerular and TI compartments were separated by differential sieving followed by enrichment of the ECM using a biochemical fractionation approach (Chapter 2, Figure 2.1) (139, 174). Using the total spectral count of ECM proteins divided by spectral count of all proteins in the cellular and ECM fractions, ECM enrichment was confirmed at the level of fractionation by MS (Figure 3.1A) with no significant differences (T-test) across the samples analysed (Figure 3.1B). Principal component analysis (PCA) revealed separation of glomerular and TI ECM samples with a little overlap (Figure 3.1C). Proteins were detected using MS and identified as present in either the TI or glomerular ECM-enriched proteome if they were found in at least 2 of the 6 samples and with a minimum of 2 spectral counts. Data were then put through HMDB to identify known ECM proteins. Using this approach, 140 and 164 proteins were identified in the TI and glomerular ECM, respectively (Appendix Table 3.1 and 3.2).
Figure 3.1 Demonstration of ECM enrichment by MS. 
A: ECM enrichment (%) derived from peptide abundance for the soluble and ECM fractions demonstrating enrichment of ECM components in the ECM fraction. B: Comparison of glomerular (G) and TI ECM enrichment in the ECM fraction, these values varied from 25-60%. C: Principal component analysis (PCA) of the ECM fractions demonstrates the separation of glomerular (ochre) and TI (green) datasets. Error bars: Mean ± SD.

3.3.2 Composition of the TI ECM of the human kidney cortex

Firstly, to gain insights into the biological function of the proteins identified in the TI ECM, gene set enrichment analysis was utilised using DAVID (https://david.ncifcrf.gov/) gene ontology (GO). The top 10 terms that contained greater than 45 proteins are listed in Figure 3.2A. The most significantly enriched GO terms were biological and cell adhesion which each contained over 60 proteins, followed by GO terms such as “extracellular structure organisation”, “extracellular matrix organisation” and “response to organic substance”. The 140 proteins identified by HMDB in the TI ECM were categorised using the database: 74 (52.9%) were identified as “ECM-associated” proteins, 30 (21.4%) as “basement membrane” proteins and the remaining 36 (25.7%)
Figure 3.2 The human kidney TI matrisome.

One hundred and forty proteins were identified in the TI ECM compartment using the HMDB. These were categorised as either other structural, basement membrane, or ECM-associated proteins. **A**: GO enrichment analysis of the TI ECM proteins. Proteins from the TI ECM list were searched using DAVID and the GO biological process annotation chart. **B**: Venn diagram showing the number of proteins identified in each ECM category as defined by the HMDB. **C**: Protein-protein interaction network showing proteins as nodes (squares) with the size of the node proportional to protein abundance (peptide intensity). The colour code represents the category of ECM proteins. Lines between nodes represent experimentally-derived and reported protein-protein interactions (Cytoscape).
as “other structural proteins” (Figure 3.2B). The TI ECM proteins with the highest number of spectral counts (>500; covered more than 50% of all spectral counts) indicative of greatest abundance, were perlecan (HSPG2), collagen I α1 (COL1A1) and α2 (COL1A2), collagen III α1 (COL3A1), collagen IV α1 (COL4A1) and α2 (COL4A2), collagen VI α3 (COL6A3), plectin (PLEC), TI nephritis antigen (TINAG) and LAM α5 (LAMA5) (Figure 3.2). A protein-protein interaction network was also generated using Cytoscape, which demonstrated the complexity of the potential networking of the TI ECM components (Figure 3.2C). The top 10 most highly connected proteins (ie. those proteins with at least 15 interactions with other proteins) in this network were: COL1A1, COL1A2, COL4A1, COL4A2, COL6A1, COL6A3, DCN, FN1, LAM γ1 chain (LAMC1), THBS1 and VTN. In contrast, 59 proteins have no known interactions. The majority of these proteins fall into the category of ECM-associated proteins (39 proteins; 66%) with 8 basement membrane proteins and 12 other structural proteins.

3.3.3 Validation of proteins identified in the human kidney TI matrix

To validate the proteomic analysis, IHC was performed for collagen I (representative of TI collagens), COL4 (representative of basement membrane collagens) and lumican (representative of proteoglycans in both TI and G compartments), three proteins detected in the TI ECM by MS. Moreover, a number of other candidates (18 proteins, namely, aggrecan, Annexin VII, Biglycan, COL6A1, COL6A2, COL6A3, Dermatopontin, EMILIN-2, FGL1, Fibronectin, FREM2, HtrA, LAM β2, LAM γ1, LAM γ3, LTBP-4, MFAP4, TIMP3) were selected for validation, however, it proved extremely difficult to find efficient antibodies for these proteins (data not shown). All three proteins were detected by IHC. In IgG controls, weak staining was observed with 10x magnification, however, this was negligible when images were zoomed to 40x. Collagen I was localised primarily to the TI spaces with low expression in the mesangial matrix and no intracellular staining (Figure 3.3A-D). Expression of collagen IV was widespread with positive staining localised to tubular basement membranes as well as other
Figure 3.3 Validation of collagen I, collagen IV and lumican expression in the cortical ECM.
Sections of normal human kidney were stained with antibodies (Chapter 2, Section 2.4) to collagen I (A, B), collagen IV (E, F) or lumican (I, J). Positive staining is indicated by a red-brown deposit (arrows). As a control for antibody specificity, sections were incubated with the appropriate IgG (rabbit IgG: C, D, G, H; Goat IgG: K, L) at an equivalent concentration to the primary antibody. Representative images are shown (n = 2 kidneys). G: Glomeruli; TI: Tubulointerstitium. Magnification: A, C, E, G, I and K: 10x; B, D, F, H, J and L: 40x.
basement membrane structures in the glomerular ECM and Bowman’s capsule (Figure 3.3E-H). The proteoglycan, lumican, was strongly expressed in both TI and glomerular matrix (Figure 3.3I-L). Lumican staining was positive in all TI matrices but predominately localised to the vascular area of the glomerular matrix (Figure 3.3J).

Validation by Western blotting was also attempted. It was hoped that it would be possible to quantify total protein in the ECM fractions of all samples by densitometry of Coomassie blue- and silver-stained gels to allow equal amounts of protein to then be run for immunoblotting. However, this was not feasible due to insufficient sample volume and/or protein degradation likely as a result of repeat freeze-thawing (data not shown).

3.3.4 Novelty of proteins identified in the human TI matrix

To examine whether the proteins identified in the proteomic analysis of the human TI ECM had been previously reported in the literature as present in the kidney ECM, the following search terms were used in PubMed (https://www.ncbi.nlm.nih.gov/pubmed): “kidney extracellular matrix”, “kidney matrix”, “kidney”, “human kidney matrix”, “human kidney” and “human”. In order to be inclusive, published results of transcriptomic profiling, RNA expression and protein localisation were all included. This analysis (Figure 3.4; Appendix Table 3.3) identified 2 proteins, caspase 14 (CASP14) and heparin cofactor 2 (SERPIND1) not previously reported to be expressed in the kidney of any species. There were also 4 proteins which have not previously been reported in the human kidney: T-complex protein 1 subunit β (CCT2), desmoglein-1 (DSG1), 2-oxoglutarate 5-dioxygenase 1 (PLOD1), and matrilin-2 (MATN2). The remaining 134 proteins have been reported in the kidney of a variety of species. Of these proteins, 59 are known to be present in the TI of a variety of species, including mouse, rat and humans, while 75 have not previously been localised to the TI ECM in any species.

3.3.5 Comparison of human kidney TI and glomerular ECM

The composition of the glomerular and TI ECM isolated from the same human
Figure 3.4 Correlation of identified TI ECM proteins with published data.
RNA profiling, RNA and protein expression studies in PubMed were used to determine the reported localisation of the 140 TI ECM proteins identified by proteomics in the human kidney. Known in TI (green) indicates the protein has previously been reported in kidney TI of human or other species. Novel in TI matrix (red) indicates the protein has not been previously localised to the TI matrix of any species. Novel in human kidney (blue) indicates the protein has not previously been identified in human kidney. Novel in kidney (black) indicates the protein has not previously been reported in the kidney of any species.
kidney (n=6) in parallel was compared using proteomics. Overall, 90% of the components identified in the TI ECM (126 proteins) were also found in the glomerular ECM, with 38 proteins specific to the glomerulus and 14 detected only in the TI (Figure 3.5A). Of the 14 proteins unique to the TI ECM, 9 were ECM-associated proteins (alpha-2-HS-glycoprotein (AHSG), annexin A13 (ANXA13), dipeptidyl peptidase 1 (CTSC), desmoplakin (DSP), neutral alpha-glucosidase AB (GANAB), endoplasm (HSP90B1), protein ERGIC-53 (LMAN1), PLEC and SERPIND1), 3 were basement membrane proteins (collagen VIII α1 (COL8A1), FRAS1-related extracellular matrix protein 1 (FREM1) and LAM α1 (LAMA1)) and 2 were categorised as other structural proteins (elastin (ELN) and kielin/chordin-like protein (KCP)). Of the 38 proteins only detected in glomerular ECM, there were 7 basement membrane proteins (LAMA2, LAMA3, LTBP4, NTN4, PAPLN, PXDN and SPARC), 6 other structural proteins (COL16A1, COL7A1, FBLN5, OGN, PRG2 and VCAN) and 25 ECM-associated proteins (Appendix Table 3.4).

In the glomerular ECM, the proportion of the 164 proteins classified using the HMDB (Figure 3.5B) as ECM-associated (90 proteins, 54.9%), basement membrane (34 proteins, 20.7%) and other structural (40 proteins, 24.4%) was similar to the TI ECM. Sixteen proteins were identified in the glomerular ECM with spectral counts over 500. Eight of these were also highly abundant in the TI ECM (COL1A1, COL1A2, COL3A1, COL4A1, COL4A2, COL6A3, PLEC and LAMA5) with the others (agrin (AGRN), COL4A3, COL4A4, COL6A1, fibrillin-1 (FBN1), LAMB2, LAMC1 and NID1) highly expressed only in the glomerular ECM. The generation of a glomerular ECM protein-protein interaction network (Figure 3.5C) showed that 96 proteins interact with other proteins while 68 proteins had no known interactions (11 basement membrane, 11 other structural and 46 ECM-associated proteins). Network analysis also revealed 16 ECM components with 15 or more known interactions. Nine of these (COL1A1, COL1A2, COL4A1, COL4A2, COL6A1, COL6A3, DCN, FN1 and LAMC1) overlapped with the most highly interacting proteins in the TI ECM. The 7 remaining highly interactive proteins in the glomerular ECM included THBS1 with 32 known interactions as well as COL6A2,
HSPG2, LAMA5, plasminogen (PLG), tenascin-C (TNC) and vitronectin (VTN) which all have 15-18 interactions.

Figure 3.5 The human kidney glomerular matrisome.
One hundred and sixty-four ECM proteins were identified in the glomerular ECM. A: Comparison of ECM proteins identified in TI (TI: Blue) and glomerular (G: Yellow) compartments. B: Venn diagram displaying the number of glomerular ECM proteins identified in each ECM category. C: Glomerular ECM protein-protein interaction network showing proteins as nodes (squares) with the size of the node proportional to protein abundance (peptide intensity). The colour code represents the category of ECM protein. Lines between nodes represent experimentally-derived and reported protein-protein interactions (Cytoscape).
3.3.6 The effect of ageing on human TI and glomerular ECM

Next, changes in the composition of the human TI and glomerular ECM as the kidney ages were explored. The kidneys were divided into two groups (young: 15-37; old: 61-69 years, n = 3 in each group). A renal pathologist blinded to sample age, examined the kidney histology of all samples (H&E-stained sections). The older kidneys generally showed mild thickening of the vessel walls, occasional focal glomerular sclerosis and TI expansion consistent with normal ageing (Figure 3.6A-D). PCA revealed separation between young and old ECM samples (Figure 3.6E). To identify specific proteins that were altered with age, hierarchical clustering analysis was performed on the spectral count data from both the TI and glomerular ECM. In the TI ECM (Figure 3.6F), 8 proteins (COL6A1, COL6A2, collagen XIV α1 chain (COL14A1), FBLN1, FN1, TGFB1, transglutaminase-2 (TGM2) and tissue inhibitor of metalloproteinases-3 (TIMP3)) were significantly up-regulated with age. Five of these proteins (COL6A1, COL6A2, COL14A1, FBLN1, TIMP3) were also up-regulated in the ECM of ageing glomeruli (Figure 3.6G). Additionally, a further 12 proteins (annexin A7 (ANXA7), BGN, COL6A3, chemokine ligand 14 (CXCL14), dermatopontin (DTP), fibrinogen-like protein 1 (FGL1), HtrA serine peptidase 1 (HTRA1), LUM, microfibrillar-associated protein 4 (MFAP4), osteoglycin (OGN), proline and arginine rich end leucine rich repeat protein (PRELP) and versican (VCAN)) were found uniquely increased in the ageing glomerular ECM. Three proteins (angiotensinogen (AGT), LAMB2 and LAMC1) were down-regulated in the old versus young glomerular ECM. To begin to validate these proteomic findings the expression of FBLN1 in two young and ageing human kidneys was examined and quantified by IFL (Figure 3.7). In the young group, Fbulin-1 (FBLN1) showed limited expression localised to the tubular basement membranes (Figure 3.7A). However, in the aged samples, there was a general increase of FBLN1 expression with strong positive staining in a few foci in the matrix but no localisation to the tubular basement membrane. In glomeruli, a similar increase of FBLN1 expression was observed in the matrix. Significant up-regulation of
FBLN1 was confirmed in both the ageing glomerular (p<0.0005) and TI (p<0.0001) compartments compared with young samples.

**Figure 3.6** Age-related changes in human TI and glomerular ECM. Sections of normal human kidneys were stained with H&E (A-D) and the histology assessed by a renal pathologist blinded to donor age. *: Thickening of the vessel wall; #: Focal glomerular sclerosis. At least 10 fields were examined for each kidney and representative images are shown. G: Glomeruli; TI: Tubulointerstitium. Magnification: 10x. E: PCA analysis of the ECM fractions demonstrates a clear separation of young and aged samples. F: TI proteins that change with age. G: G proteins that change with age. Red: High expression; Blue: Low expression. Numbers at the top of the heat maps indicate the donor age in years. Z score: Fold-change in protein expression.
A: Sections of normal human kidney were stained with an antibody to FBLN1 and antibody binding visualised with an Alexa Fluor 568-conjugated secondary antibody (red), nuclei were stained with Hoechst 33342 (blue). Control sections were incubated with mouse IgG at an equivalent concentration to the primary antibody. Representative images are shown (n=2 kidneys/group: Young: 15 and 29 years old; Aged: 61 and 67 years old. White circles: glomeruli; G: Glomeruli; TI: Tubulointerstitium; arrows indicate positive antibody binding. Magnification: Left panel 20x, right panel 40x. B: The fluorescent signal was quantified using ImageJ. IgG controls show no signal, therefore could not be quantified. ***: P < 0.0005. ****: P< 0.0001. N=40 randomly distributed fields per kidney section. Corrected Total Fluorescence = Integrated Density - (Area of selected cell x Mean fluorescence of background readings). Error bars: Mean + SD.

3.4 Discussion

To date, the ECM of the normal kidney TI has largely been investigated using candidate-based approaches. Here an unbiased comprehensive global approach to study the human TI matrix using MS-based proteomics was applied and validated by IHC/IFL. Before identifying proteins using MS, a previously published biochemical protocol (139) was used to enrich ECM components for glomeruli and TI in order to improve the identification of matrix proteins by reducing the amount of cellular components. Inevitably, some cellular components remain in
enriched samples as the enrichment is about 40% (Figure 3.1), suggesting that it is necessary to use a matrix protein-defining database for matrix protein identification. Therefore, the human matrisome database (HMDB), a bioinformatics-based database which includes structural ECM proteins and proteins that can or may interact with or remodel ECM, was used to filter out cellular proteins and maximise the identification of matrix proteins. Traditionally, researchers have used the HMDB to identify matrix proteins, but one needs to be aware that the HMDB is a constructed database based on bioinformatics. As a consequence, it may contain some hypothetical matrix proteins which possess the domains to interact with/remodel matrix but are not actually present in the ECM.

The TI ECM proteome was compared to that of glomeruli isolated in parallel from the same kidneys. In addition, the ECM proteomes from both compartments were compared in young and old kidneys to identify age-related changes which may be relevant to changes in cell function with age and to chronic kidney disease (CKD) which is often regarded as premature ageing (264). Both normal ageing of the kidney and CKD are characterised by scarring and accumulation of ECM, thus an understanding of how the ECM changes with age might also provide insights into the pathological changes in CKD. These results indicate that despite the differences in cell types, function and matrix structures between the TI and glomeruli, the content of TI ECM is similar to the glomerular ECM (139, 167) which might mean that diverse cell types produce a very similar core set of proteins to fulfil the basic functions, such as structural support and cell attachment, and the specialisation of matrix and cell-matrix interactions may come from a relatively small number of proteins. This hypothesis was further confirmed by the study of age-related changes which indicates that only about 10% of ECM proteins change with age in both the TI and glomerular ECM. One limitation of this study is that the number of samples is small (n=6 for categorising TI and glomerular ECM, n=3 in the young and old groups). In addition, the categorisation of the young kidney group is wide (for 15 to 37 years of old). This is due to the relatively small number of young human kidneys available for transplantation and those that are available are less likely to unsuitable for use.

3.4.1 Composition of human TI matrix
Proteomics is an unbiased high-content method to analyse complex samples (265). By the nature of MS-based proteomics, there is no need to predetermine the target such as is required for candidate-based approaches (266). In this study, 140 matrix proteins were identified in the TI ECM proteome, of these 59 proteins were already known to be present in the TI though not necessarily in the human TI, while the remainder were all new to the TI. The number of proteins highlights the previously unrecognised complexity of the TI ECM. However, it should be noted that proteomics does not permit ascribing proteins to particular matrix structures for example, the tubular basement membranes or the interstitial matrix.

An initial search of all identified proteins in the UniProt database for tissue localisation yielded very limited data. Therefore, another search was performed using Pubmed and searching the protein names with different key phrases to identify tissue compartment, localisation and species-specificity of each protein. This search revealed 2 proteins that had not previously been identified in the kidney of any species and 4 proteins that had not previously been identified in the human kidney while the remainder of the proteins (134) had been reported in human kidney, but of these only 59 had previously been localised to the TI. Thus 75 proteins, were newly identified as present in the TI ECM. By HMDB category these include 46 ECM-associated proteins, 21 other structural proteins and 8 basement membrane proteins. This study confirmed data from candidate-based approaches on localisation of 59 proteins in the renal TI (Figure 3.4, Appendix Table 3.3). Among the 134 known and newly identified human TI ECM proteins, several families are represented by multiple members including the COLs, ANXAs, CTSs, SERPINs and LGALSs. The collagens (19) are the biggest protein group and include the well-characterised collagens (collagen I, III, IV, V and VI), as well as some less well-characterised collagens, such as collagen XII, XIII, XV and XVIII (57, 267-270). Eight of the 19 collagens identified in this study had previously been reported in the TI. For example, COL1A1, COL4A1, COL4A2 and COL4A6 were previously reported in mouse kidney TI ECM and in human proximal tubular epithelial cells (52, 271-273). This study, provides the first evidence for 11 other collagen isoforms in the TI ECM including
other isoforms of collagen IV (COL4A3, COL4A4 and COL4A5) and 3 isoforms of collagen VI (COL6A1, COL6A2 and COL6A3). Interestingly these isoforms have recently been reported in the human G ECM (139, 167) suggesting some commonality between the TI and glomerular matrices.

The Annexins (ANXA1, 2, 4, 5, 6, 7, 11 and 13) are the second largest group of proteins identified in the human TI ECM. Annexins bind phospholipids in a Ca$^{2+}$-dependent manner. Although more commonly associated with intracellular functions some extracellular annexins have been identified in the human placental matrix (ANXA1, 2, 4 and 5) (274) and human G ECM (ANXA5, 6, 7 and 11) (139, 167). In addition, ANXA1, 2 and 4 were shown to be expressed by renal epithelial cells of various species in culture suggesting tubular epithelial cells might be the source of ANXA1, 2 and 4 in TI ECM (275-277). RNA for ANXA13 has been detected in human kidney tissue (278), our data suggest that Annexin 13 is present in the human kidney ECM and could be potentially an extracellular annexin.

The cathepsins, CTS, are another large family of proteases initially identified in lysosomes (279). In this study, 7 cathepsins were detected in the TI ECM which could be classified into three subfamilies: serine (cathepsin A and G), cysteine (cathepsin B, C, H and Z) and aspartate (cathepsin D) cathepsins. All of 7 have previously been reported in the human kidney, and 5 cathepsins (A, B, D, G and Z) were identified in human G ECM (139, 167, 280, 281). Our data confirmed the presence of cathepsin B, D and H in the TI ECM as they were reported to be produced by cultured human and rat proximal tubular epithelial cells (282-284) however, but this is the first report localising cathepsin A, C, G and Z to the human TI matrix.

The serpins are a superfamily of serine protease inhibitors (285). This study identified 7 serpins in the human TI ECM, 6 of which (Serpin A5, B12, C1, F1, G1 and H1) had previously been reported in the human kidney (139, 286-291). Five of these 6 (A5, B12, C1, G1 and H1) serpins were also found in the human G ECM (139, 167). SERPINA5 and H1 have been described in human proximal tubular epithelial cells and in mouse tubulointerstitial fibroblasts (288, 292, 293). This study suggests localisation of
serpin B12, C1, F1 and G1 to the human TI ECM and provides the first evidence for serpin D1 in the kidney of any species.

The galectins (LGALSs) are a family of 15 lectins, with diverse extracellular and intracellular functions (294). Four galectins (1, 2, 3 and 8) were identified in the human TI ECM. Of these, LGALS1 and LGALS3 have been reported in cultured human and mouse renal epithelial cells (295-298), and LGALS8 has been localised to the human kidney (299). One study suggested that LGALS3 protects renal tubules as after UUO there was more tubular damage with 21% increased collagen deposition in LGALS3-deficient mice compared with wild-type controls (300). The only galectin newly identified in the human TI ECM in this study is LGALS2, which has previously been detected in the plasma of renal transplantation patients and proposed as a marker of renal recovery (301).

Taken together the data from these 5 major protein families are consistent with published candidate-based studies localising particular proteins to the kidney (52, 139, 271-273, 275-277, 286-290). Importantly our data provide evidence for the TI localisation of 75 proteins previously reported as present in the human kidney but not ascribed to a particular compartment, and contribute to a better understanding of the composition of the ECM in this tissue compartment.

In addition to the 134 proteins previously identified in human kidney, this study revealed 2 proteins (CASP14 and SERPIND1) not previously reported in the kidney in any species and 4 proteins (CCT2, DSG1, MATN2 and PLOD1) not previously reported in the human kidney (Figure 3.4, Appendix Table 3.3). CASP14, a non-apoptotic caspase, has been implicated in a range of biological functions (eg. keratinocyte differentiation (302) and protein processing (303)), and has been identified in human oral carcinoma (302) and diabetic oedema (304). The investigators suggested that CASP14 may contribute to the formation of the skin’s permeability barrier (303). The function of CASP14 remains to be established in the kidney, however, its function might relate to those identified in skin, such as, cell differentiation and protein processing. SERPIND1, also known as heparin cofactor II, is a thrombin co-inhibitor which can be activated
by heparin and glycosaminoglycans (305). Previous studies identified SERPIND1 mRNA in human liver (306) and SERPIND1 protein expression in ovarian tissues (307). In addition, other investigators showed that SERPIND1 is secreted by fibroblast and smooth muscle cells, and deposited in the vascular matrix (308). There is also evidence showed that SERPIND1 can attach to matrix proteins such as biglycan and decorin (309). It could be speculated that SERPIND1 might be deposited in the vascular matrix of the kidney and its role in the kidney might be involved in preventing formation of thrombi. However, the detailed roles of these 2 novel proteins (CASP14 and SERPIND1) in the kidney remain to be established. Of the 4 proteins novel to the human kidney (Figure 3.4, Appendix Table 3.3), MATN2 is categorised as an other structural proteins while the rest are ECM-associated proteins. MATN2 is a well-known ECM protein which is widely distributed in mouse tissue and in the mouse kidney is expressed in the glomerulus and distal convoluted tubular basement membrane (310). It has been shown that MATN2 not only binds to itself but also interacts with COL1, FBN-2 and FN suggesting it is important in forming supramolecular ECM structures (311). MATN2 expression is also up-regulated by high glucose and TGF-β1, and suppressed by TGF-β1/Smad3 inhibitors in mouse mesangial cells (312). Taken together these data suggest MNT2 may have an important role in ECM organisation and is regulated by growth factor signalling in the kidney. CCT2, also called TCP-1β, is the β subunit of chaperonin-containing t-complex polypeptide 1 (313). In a type 2 diabetic mouse model, increased expression of CCT2 was detected in the kidney (314), and urinary CCT2 was found to be associated with the hyperfiltration phase in type 2 diabetic patients compared with healthy controls (314). CCT2 also regulates high glucose-induced proliferation, migration and contraction of mouse mesangial cells by changing the filamentous action/globular actin ratio (315). It has been suggested that CCT2 may be highly associated with type 2 diabetes, however, the role of CCT2 in normal Ti ECM is still unclear. DSG1 was identified as an ECM protein and is categorised as ECM-associated protein. This might be because most published literatures suggest that its functions are associated with cell-cell (316, 317) or cell-matrix adhesion (318) and it might be involved in matrix degradation (319). Therefore, DSG1 in the Ti matrix may bind to/remodel the renal matrix. PLOD1, procollagen-lysine, 2-oxoglutarate 5-
dioxygenase 1, also known as lysyl hydroxylase 1, is a collagen-crosslinking enzyme (320) reported to be expressed in mouse kidney (321) and now in the human kidney TI ECM. It seems likely that in the kidney, PLOD1 is involved in the formation of supramolecular collagen structures and could affect the stiffness of matrices which, in turn, influences cell function.

In all proteomic studies validation of at least some of those identified proteins by an alternative method is necessary (possibility of false discovery). Collagen I staining (Figure 3.3B) confirmed its primary localisation in the TI consistent with it being a well-known component of the kidney interstitial matrix (11). Another well-established collagen the basement membrane protein, collagen IV, was shown to be present in the basement membranes of glomeruli and tubules (Figure 3.3F) which is consistent with previous reports (11, 322). Lumican was also selected for validation as it is representative of other structural proteins present in both TI and G compartments with reasonable abundance (Figure 3.2 C and Figure 3.5C). Our results are consistent with those of Fridén et al. and Schaefer et al., suggesting lumican is located in the TI and glomerular capillary matrix (323, 324).

3.4.2 Comparison between human TI and G matrix

Global profiling of the TI ECM reveals an ECM comprised of 140 proteins. Comparison with the G ECM proteome from the same kidneys analysed in parallel revealed a high degree of similarity between the ECM of the two compartments with 126 ECM proteins common to both TI and G ECM (90% and 77% of the total ECM proteomes, respectively suggesting a common core matrisome). The shared group contains 65 ECM-associated proteins, 34 other structural proteins and 27 basement membrane proteins (Appendix Table 3.1 and 3.2). There were comparatively few proteins unique to the two compartments, 14 proteins (10% of the total) in the TI and 38 (23%) in G. These differences may reflect differences in specialised functions in the two compartments. Among the 14 proteins that were only detected in the TI ECM, there are 3 basement membrane proteins, 2 other structural protein and 9 ECM-associated proteins (Appendix Table 3.1). Five of these proteins, including 2 basement membrane proteins (COL8A1 and LAMA1, reported in human kidney) (325, 326) and 3 ECM-associated proteins
(DSP (identified in MDCK cells) (327, 328), HSP90B1 (reported in human proximal tubular epithelial cells) (329), and PLEC (reported in rat and human tubular epithelial cells) (330, 331)) have previously been identified in the renal TI of a variety of species. Eight of the proteins have not previously been described in the TI ECM: 1 basement membrane protein (FREM1), 2 other structural proteins (ELN and KCP) and 5 ECM-associated proteins (AHSG, ANXA13, CTSC, GANAB and LMAN1). The results indicated that 14 TI ECM-only proteins covered a range of categories and were not confined to any particular groups. One ECM-associated protein, SERPIND1, was only detected in the TI ECM and has not previously been reported in the kidney in any species; its function in the kidney remains to be elucidated.

Among the 38 proteins only detected in the G ECM, there are 7 basement membrane proteins, 6 other structural proteins and 25 ECM-associated proteins (Appendix Table 3.4). A comprehensive web-based search using the keywords ‘kidney’ and ‘glomeruli’, revealed that 22 of the 38 proteins have previously been reported in the glomeruli of a variety of species. Most of the information on the 22 ECM proteins already reported in glomeruli was derived from two proteomic studies of G ECM matrix (139, 167) which highlighted the high content advantage of proteomics and the important contribution of this technique in furthering the understanding of the ECM. Eleven proteins in the G ECM were identified previously in the kidney (but not localised to glomeruli) including 2 basement membrane protein (LTBP4 and PXDN), 4 other structural proteins (COL7A1, FBLN5, OGN and PRG2) and 5 ECM-associated proteins (ADAM9, CTSS, CXCL14, PLOD2 and SERPINF2). There were 5 proteins identified in the G ECM (1 other structural protein (COL16A1), 4 ECM-associated proteins (ADAMTSL5, FGL1, PLSCR1 and PRG3)) which were novel to the kidney (Appendix Table 3.4; italics). Similar to ECM proteins only identified in TI, the 16 proteins newly identified in the human G ECM covered a variety of protein categories and were expressed at a relatively low level (highest was 37 spectral counts) which may explain why they have not been identified previously.

3.4.2.1 Comparison of G ECM proteins identified in this study with previously published datasets
The glomerular ECM proteome obtained was also compared with those from previous studies (139, 167). In the present study, the number of proteins detected in the glomerular fraction was higher than in previous studies (an increase of ~14%) most likely to due to advances in the MS detection systems, MS data analysis software and sample size. Of the 164 proteins identified in the G ECM, 77 proteins were also identified by Lennon et al. (139). These included the majority of the core matrisome proteins reported by these investigators (21 out of 24 other structural proteins and 23 out of 24 basement membrane proteins) (139). Our study identified 18 additional other structural proteins (COL14A1, COL16A1, COL5A1, COL5A2, COL7A1, ECM1, EFEMP1, FBLN5, IGFBP7, LUM, MATN2, MFAP4, MFGE8, OGN, PRG2, SBSPON, VCAN and VWF) and 8 basement membrane proteins (LAMA3, LAMA4, LTBP4, NTN4, PAPLN, PXDN, SPARC and TNC). In addition to basement membrane and other structural proteins, our study also identified 90 ECM-associated proteins. However, the major difference between our study and that by Lennon et al. (139) was in the ECM-associated proteins with only one third of the proteins (33 out of 96) previously identified found in the present study. Although the two studies used the same fractionation technique, differences may due to the increase of number of kidneys (from 3 to 6 kidneys) and improvements in instrumentation and software (139). Comparison of our study with the analysis of Hobeika et al. (2017) showed that similar other structural proteins (17 proteins) and basement membrane proteins (21 proteins) were identified in both studies and again, the major variation was in the ECM-associated proteins (containing ECM regulators, secreted factors and ECM-affiliated proteins) with only 56 of the 109 previously identified proteins seen in the present study. When comparing the studies of Hobeika et al. and Lennon et al., 91 proteins were identified in both, 56 proteins were only identified in the Hobeika study (2 new LAM isoforms, LAMA3 and LAMA4, and 54 ECM-associated proteins including Lumican (167)), 53 proteins were only identified in the study by Lennon et al. (7 basement membrane proteins, 5 other structural proteins and 41 ECM-associated proteins) (139, 167). Of note, sample preparation and analysis used by Hobeika et al. differed from our study and that of Lennon et al. in that glomeruli were isolated from histological sections by laser-capture microdissection rather than by
differential sieving and different enrichment method, analytical machine (LC-MS system) and software were used, which could affect the proteins identified (139, 167, 332-334). In summary, although there are some variations in ECM-associated proteins when using proteomics to identify proteins, the core human G matrisome identified in three independent studies is very similar. It may be speculated that this core of matrix proteins is less sensitive to differences in glomerular isolation and matrix enrichment methods and represents a core which is essential in glomerular matrix.

3.4.3 Changes of the kidney ECM matrix in ageing

The availability of human kidney samples over a range of ages allowed us to compare the G and cortical TI ECM from two groups defined as young (15, 29 and 37 years of age) and old (61, 67 and 69 years of age), to examine age-related changes in the TI and G ECM. It is perhaps surprising that there were not more changes with age (28 proteins), however, other studies looking at the changes in transcriptomics (all RNA transcripts) in human kidney (37 genes) and changes of proteins in male (22 proteins) and female (33 proteins) mouse kidney showed a similar number of age-related changes (173, 335). In the human TI, 8 proteins showed a positive correlation with ageing, while in glomeruli, the expression of 17 proteins increased with age and 3 proteins (AGT, LAMB2 and LAMC1) declined (Figure 3.6). There were 5 proteins present in both glomeruli and TI, which increased with age: COL6A1, COL6A2, COL14A1, FBLN1 and TIMP3. There are 6 different α chains of collagen VI, the genetically distinct chains COL6A1, COL6A2 and COL6A3, and the newly identified more homologous COL6A4, COL6A5 and COL6A6 chains (336). Increased expression of collagen VI has been reported in cultured mesangial cells exposed to high glucose (337) as well as in other fibrotic settings such as muscle fibrosis (336) which may suggest that the increased expression of COL6A1 and COL6A2 reflects age-related fibrosis. COL14A1, also known as undulin, has been detected in autosomal dominant polycystic kidney disease (ADPKD) kidneys (338, 339) and is elevated in the later stage of hepatic fibrosis (340) again suggesting potential involvement in age-related fibrosis in the kidney. FBLN1 is a secreted glycoprotein which binds other ECM proteins such as FN and LN (341). FBLN1 suppresses the migration of many cancer cells which may prevent cancer cells
including renal carcinoma cells, from metastasising, via its FN-binding ability (341-343). FBLN1 expression is increased in hepatic (344) and renal fibrosis (345). For example, in CKD and diabetic patients, increased plasma FBLN1 was observed compared with age-matched healthy controls (346). FBLN1 showed a positive correlation with serum creatinine and an inverse correlation with glomerular filtration rate (346). Another study also reported a significantly high level of plasma FBLN1 in patients with glomerulonephritis (347). These studies suggest that FBLN1 could be used as a marker for CKD or kidney damage, thus an increase in FBLN1 in the ageing TI and G may reflect age-related fibrosis. Three members of the TIMP family, TIMP1, 2 and 3 have been identified in the human kidney however only TIMP3 is reported to be up-regulated with age (11). Conversely decreased TIMP3 expression was observed in the fibrotic mouse kidney (348). Moreover, Wang and colleagues demonstrated that increased TI fibrosis was observed in TIMP3 knock out mice after UUO compared with wild-type controls (98). Another study showed that TIMP3 deficiency leads to chronic TI fibrosis in aged mice (24-month) compared with age-matched wild-type controls mice (349), suggesting TIMP3 may have a protective role in the kidney. TIMP3 can affect ECM turnover either via inhibition of proteases such as ADAM17 and MMPs, or via controlling the transition of pericytes to myofibroblast-like cells (350, 351). Thus, the increase in TIMP3 observed with age in the human kidney is perhaps unexpected but may be part of a protective mechanism against age-induced fibrosis.

In addition to the 5 proteins showing age-related changes in both the TI and G ECM, 3 proteins increased with age only in the TI: FN1, TGFBI and TGM2. FN1 is a glycoprotein that binds to collagens and to cell surface matrix receptors and is involved in cell-cell and cell-matrix adhesion, cell migration and wound healing (352-358). An age-related increase of FN/EDA-FN (fibronectin with an extra EDA domain) was reported in the mouse kidney (359) and in the rat TI ECM (137). Our data are consistent with published studies and confirmed an age-related increase of FN expression in human TI ECM. TGFBI, TGF-β1-induced protein ig-h3 also known as BIGH3, regulates cell adhesion and proliferation (360). An age-dependent increase of TGFBI mRNA expression was reported in the mouse heart (361). In the kidney, there was increased expression of TGFBI protein in mice fed a high-fat diet and in vitro TGFBI increased apoptosis.
of human renal proximal tubular epithelial cells in vitro (362). However, to date there have not been any studies of TGFB1 expression and function in the ageing kidney. TGM2, transglutaminase 2, is a ubiquitously expressed multifunctional protein involved in crosslinking ECM proteins (363-365). Similar to our study, a 2-4 fold increase of TGM2 expression was reported in the aged mouse kidney (24 months old) together with increased crosslinking of matrix proteins and fibrosis, compared with 6 months old animals and interstitial fibrosis was induced by subcapsular injection of TGM2 into the kidney of young mice (87). This observation was confirmed in another study using mice subjected to UUO or aristolochic acid nephropathy, which showed increased TGM2 levels associated with tubulointerstitial fibrosis (135). Moreover, after UUO, there was a significant reduction in interstitial fibrosis with less collagen deposition in TGM2 knock out mice compared with kidneys from wild-type controls (366). Similar to the kidney, an age-dependent upregulation of TGM2 expression has been reported in allergic airway inflammation in mice (367). Taken together these data suggest that the age-related increase in TGM2 in the human TI ECM reflects age-related fibrosis in the human kidney (Figure 3.6C).

In the G ECM, 12 proteins were increased with age. Eight are categorised as other structural proteins (VCAN, COL6A3, MFAP4, DPT, LUM, BGN, OGN and PRELP) and 4 as ECM-associated proteins (FGL1, ANAX7, CXCL14 and HTRA1). VCAN is proteoglycan which has been linked to a variety of renal pathologies (368, 369) suggesting that VCAN may be involved in age-related fibrosis in the human kidney, although data from diabetic mouse kidneys in which VCAN declines with disease, may (370) suggest that there is not a simple linear relationship between VCAN and disease. HTRA1 is a member of HTRA serine protease family and expressed in normal human breast epithelium, epidermis, liver and the kidney cortex (371). Many studies suggest that HTRA1 inhibits the signalling of TGF-β, however, the detailed mechanism is still unclear (372). It also be shown that HRTA1 can bind to TGF-β family proteins, such as activin and Bone morphogenic protein 4 (BMP4) (373). However, in contrast, a recent study demonstrated that LTBP-1 is a target of HTRA1, suggesting that in human skin fibroblasts, HTRA1 can facilitate TGF-β signalling (374). Moreover, HTRA1 can degrade ECM proteins, such as collagen II and FN (375). Thus,
the role of HTRA1 in TGF-β signalling and fibrosis is unclear. In this study, the role of increased HTRA1 in the ageing human kidney is similarly unclear in that it may facilitate the complex regulation of TGF-β signalling to drive a fibrotic response and/or modulate ECM protein turn-over.

Surprisingly few proteins decreased with age with only 3 proteins down-regulated in the G ECM, 2 of which are LAM isoforms (LAMB2 and LAMC1). LAMB2 is a key component of the GBM and mutation of this gene causes glomerular disease and renal failure in mice and humans (376, 377). For example, in LAMB2 deficient mice, severe proteinuria developed with failure of glomerular filtration barrier formation (378). Another study showed that loss of LAMB2 expression was detected in the patients with presence of congenital nephrotic syndrome and microcoria (Pierson syndrome) (379). These data suggest that decreased LAMB2 may be associated with damage in GBM in the ageing kidney. Although increased level of LAMC1 has been reported in patients with type 1 diabetic nephropathy (380, 381), a recent study showed that there was an increase in the turnover of LAMC1 in CKD patients (382). It could be speculated that the increase in turnover of LAMC1 could take place in ageing kidney, since there are some similarities shared between ageing and CKD (383, 384), which means less LAMC1 could be observed in ageing kidney matrix. The third protein which decreased with age in the G ECM was AGT. AGT is cleaved by renin to produce angiotensin I and then converted to angiotensin II, a vasoconstrictor which regulates aldosterone secretion, as well as water and salt absorption (385). Increased angiotensin II has been widely implicated in fibrosis (386-389). Thus, an increase in AGT with age might have been expected however it is possible that in normal ageing downregulation of AGT may form part of a protective mechanism against age-related hypertension and/or fibrosis. Overall, the up- and down- regulation of proteins in the old G and T1 ECM compartment highlights complex changes that can occur in the ageing human kidney and the role of these proteins in the ageing process warrants further investigation.

One limitation of the proteomic analysis is that as the name suggests, this analysis focusses on the protein components of the ECM, thus non-protein components
were not identified in this characterisation of the Human TI and G matrix. Although the protein core of proteoglycans and lipoproteins can be identified by proteomics, this analysis does not provide into changes in the associated GAGs and lipids. As reviewed in Chapter 1, non-protein matrix components have been shown to be altered in CKD and it is likely there are age-related changes in non-protein components in the ECM. A variety of techniques could be used to examine gross changes of non-protein components in ageing TI or G matrix, including Alcian blue staining (for acidic GAGs), sGAG assay (for sulphated GAGs) and Oil red O staining (for lipids). For example, Alcian blue staining was used to show a positive correlation between increased sulphated GAGs and ECM accumulation in patients with end stage renal disease (390). In contrast, there was a age-related decline in sulphated GAGs in rat kidney (comparing 6-week-old with 2-year-old) (391). Although sulphated GAGs initially increased between 2 month to 3 years in monkey kidneys, there was a decline between 3-year-old and 12-year-old (392). Oil red O staining has been used to show the accumulation of lipid droplets in the kidney matrix of mice fed a high fat diet (110). These data suggest that non-protein component also alter with ageing in the kidney matrix.

3.4.4 Conclusion

In conclusion, global profiling of the human TI ECM identified 140 proteins of which 2 have not previously been identified in the kidney, 4 are novel to the human kidney and 75 have not previously been localised to the TI matrix. Protein interaction network analysis revealed 56 proteins with no known interactions i.e. proteins for which there are no published interactions. These proteins are likely less well studied and their role in the kidney remains to be established. Comparison of the human G and TI ECM illustrated the complexity of matrices in the two compartments and revealed a large group of shared proteins as well as proteins unique to each compartment. Finally, comparison of the human TI and G ECM in young and old kidneys showed age-related changes in the ECM highlighting the potential importance of understanding the ageing process and the functional consequences of changes in the ECM. Of note, this is the first comprehensive analysis of the human TI matrix, a more complete understanding of the matrix
in both normal young and old kidney will underpin the understanding of pathological changes in the matrix associated with a variety of different renal diseases and the consequences of those changes on tissue function.
Chapter 4

Development of a decellularisation protocol for adult human kidney cortical tissue and characterisation of the decellularised ECM
4.1 Introduction

In Chapter 3, the ECM of the human TI and G was characterised and demonstrated a complex matrix in both compartments. ECM plays a key role in regulating cell and tissue function (80). One approach to studying the regulatory role of the matrix has been to examine the behaviour of cells on decellularised tissue scaffolds (393, 394). Such scaffolds have the advantage of retaining the composition and ultrastructural features of the parent tissue. In the kidney, the central role of changes in the tubulointerstitium in the pathogenesis of CKD and progressive scarring of the kidney (269, 395, 396) has focused attention on the cortex. This chapter describes the development of a protocol to decellularise human kidney cortex and the characterisation of the decellularised matrix. The following chapter (Chapter 5), details studies of recellularisation of the scaffolds to explore the interaction between the different human renal cell types with the kidney ECM.

As reviewed in Chapter 1, kidneys from multiple species have been decellularised using a variety of chemicals, detergents and enzymes. For example, mouse, rat, pig and human kidneys have all been decellularised using detergents (196, 219, 220, 224, 229, 232-234, 236). Although most decellularisation studies have used fresh tissue, frozen tissues (stored at either -20 or -80°C) have also been used successfully with the advantages of allowing decellularisation of archived tissue and the ability to collect tissue samples which can then be analysed at the same time (211, 220, 223). The work described in this chapter used tissue from a number of different human kidneys which had been sliced and stored in -80 °C on receipt (the kidneys had been donated for transplantation but were deemed unsuitable for transplantation (Chapter 2, Section 2.2)).

Almost all decellularisation studies use water or PBS as the first step in the decellularisation process to wash the tissues, eg. to remove blood (193, 196, 216-221, 223, 233, 234). In this chapter, as the human kidneys used was already perfused (described in Chapter 2, Section 2.2), water was selected for the initial step in developing our decellularisation protocol as it will rupture the cell membrane by osmotic pressure.
Different chemicals and detergents have then been used to decellularise kidneys both by perfusion and in tissue cube-based methods. The success of decellularisation is generally assessed in terms of the efficiency of removal of cellular materials and preservation of the matrix. Different investigators have reported different data on the widely used detergents even when decellularising the same tissue (193, 196, 216-221, 223, 233, 234). Comparing SDS and Triton for decellularisation of pig kidney tissue cubes, Choi et al. suggested that Triton provided effective decellularisation with good preservation of the matrix (222). In contrast, results obtained by Poornejad et al., suggested that Triton failed to remove all cellular components from pig kidney tissue cubes and that of the 5 reagents tested (NaOH, PAA, SDS, Triton, Trypsin/EDTA), SDS provided the best for decellularisation (220). Another study also showed that SDS could effectively decellularise whole pig kidney by perfusion (223). In a study of perfusion decellularisation of the whole rat kidney, a combination of Triton and SDS was suggested to provide the most efficient decellularisation (196). However, He and colleagues have suggested that prolonged exposure to SDS can cause damage to the matrix (216) and that exposure to this detergent should be limited. They also showed that 4 hours of SDS treatment could effectively remove all cellular components from whole rat kidneys decellularised by perfusion (216). In terms of primate or human kidney, whole human kidneys (233, 234) and slices of monkey kidney (232) have been decellularised using SDS. In many protocols, detergent treatment is followed by DNase treatment to remove any residual DNA. For example, DNase treatment was used after SDS and/or Triton treatment in decellularising whole human/pig kidneys and pig kidney cubes (162, 221, 222, 230, 231, 233). Although there are both advantages and disadvantages to using SDS and Triton in decellularisation, both have been shown to achieve efficient decellularisation of kidney tissue and were selected as candidates for detergent treatment for the development of new protocol.

In the present study, a cube-based decellularisation approach was adopted to generate scaffolds for in vitro use to study matrix regulation of cell behaviour in the kidney.
The added advantages of using tissue cubes are that use of individual kidneys can be maximised, since only a small amount of tissue is required, multiple variables can be tested on the same tissues and scaffolds from different kidneys can be compared. Human kidney cortex cubes (5x5x2.5mm) were decellularised using water followed by detergent washes and DNase treatment. H&E staining, residual detergent and DNA quantitation, TEM and LC/MS-MS were used to determine the efficacy of decellularisation and the ultrastructure and composition of the ECM scaffolds. The optimised protocol achieved sterile ECM scaffolds with complex composition and good ultrastructural preservation in ~14 hours. Our optimised protocol is shorter than the study by Nagao and colleagues (decellularised human kidney cubes 1x1x1cm for 10 days) with less exposure to detergent (168).

4.2 Materials and Methods

4.2.1 Decellularisation of human kidney cortex tissue cubes

This section describes the optimised protocol for decellularisation. The variables tested to develop the final protocol, including ionic and non-ionic detergents, as well as the different number and duration of wash steps, are described in the Results (Section 4.3). The six human kidneys used in this chapter were from donors aged >60 years (details in Chapter 2, Table 2.1). All water used in this protocol was reverse osmosis water (RO water; Millipore, Watford, UK), unless otherwise stated.

Slices (5-10mm thick) of archived decapsulated human kidney cortex stored at -80°C (Chapter 2, Section 2.2) were cut into cubes (5mm x 5mm x 2.5mm) on a pre-cooled metal plate using a scalpel (Swann Morton 22, Sheffield, UK). Six cubes were placed in a 50ml centrifuge tube (Corning) in 35ml sterile water and shaken for 30 minutes on a Linear Shaker (Fisher, Rochford, UK) at 170 rpm at room temperature. The water was changed 7 times at 30-minute intervals. After the final wash, 30ml 1% SDS was added and the samples shaken for 1 hour at 170 rpm at room temperature followed by a 20-minute wash in 20ml water. This sequence was repeated 4 times. After the final
water wash, cubes were rinsed 3 times, 10 minutes each, with shaking at room temperature, in 20ml PBS to remove residual SDS. Aliquots (1ml) of each wash solution were collected to estimate the SDS concentration. DNase I solution (0.0225 mg (75.67 Kunitz Units)/ml, in 1x reaction buffer (0.5mM CaCl$_2$, 2.5mM MgCl$_2$ in MilliQ water (18.2MΩ)) was added to the samples and incubated for 2 hours at 37°C in a hybridization oven (Stuart Scientific, Staffordshire, UK) rotating at 10 rpm to remove residual DNA. Cubes were then washed 3 times, 20 minutes each, in 20ml of water with shaking at 170 rpm, at room temperature. Decellularised cubes were assessed for residual detergent, residual DNA and histology.

![Figure 4.1 Schematic of the optimised decellularisation protocol.](image)

**4.2.2 Measurement of SDS in decellularised cubes**

As the subsequent aim was to determine the effect of human kidney ECM on cell function and behaviour (Chapter 5), it is important to remove SDS which might adversely affect cell viability. Residual SDS in the decellularised cubes was measured as previously described (397). Samples (70µl) from the 3 final water wash steps were mixed with 500µl methylene blue solution (0.005% methylene blue solution in 0.7mM sodium phosphate, pH 7.2). Chloroform (3ml) was then added and the samples vortexed for 7 seconds. All samples were centrifuged at 600 x g for 3 minutes at 4°C to separate the aqueous and organic phases and incubated for 10 minutes at room temperature. Samples (200µl, in triplicate) were transferred into individual wells of a 96-well plate (Corning) and the absorbance read at 650nm on a spectrophotometer (Biochrom EZ Read 400, Cambridge, UK). Detergent percentage (w/v) in the tissue samples was determined from a standard curve 0-0.02% SDS (w/v).

**4.2.3 Measurement of DNA in decellularised cubes**
A DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) was used to extract DNA from 5-25mg of decellularised tissue to measure residual DNA. Ethanol was added to Buffer AW1 and AW2, according to the manufacturer’s instructions. Tissue samples were put into individual 1.5ml microcentrifuge tubes (ThermoFisher), 20μl proteinase K and 180μl Buffer ATL were added and mixed by vortexing. Samples were incubated at 56°C for 20–40 minutes with occasional vortexing during the incubation until digestion was complete i.e. no tissue was visible in the digestion solution and the solution looked clear, and then vortexed for 15 seconds. After vortexing, 200μl Buffer AL was added to each sample, mixed by vortexing, and incubated at 56°C for 10 minutes. Absolute ethanol (200μl) was added and mixed by vortexing. The mixture was transferred onto DNeasy mini spin columns placed in 2ml collection tubes. Columns were centrifuged at 6,200 x g for 1 minute at room temperature and transferred to clean 2ml collection tubes, Buffer AW1 (500μl) was added and columns washed by centrifugation at 6,200 x g for 1 minute at room temperature. After centrifugation, columns were transferred to clean 2ml collection tubes, 500μl Buffer AW2 added and centrifuged at 20,000 x g for 3 minutes at room temperature to wash the columns again. The collection tube and flow-through were discarded. The column was then placed in a 1.5ml microcentrifuge tube and 200μl Buffer AE added, incubated for 1 minute at room temperature and centrifuged at 6,200 x g for 1 minute to elute the DNA from the spin column membrane. The DNA concentration was measured at 260nm on a Nanodrop ND 8000 (ThermoFisher). The 260/280 ratio was used to determine levels of protein contamination.

4.2.4 Histology of decellularised tissue cubes

Decellularised cubes were fixed in 4% paraformaldehyde (PFA; made from stock 16% PFA (TAAB Laboratories) with PBS (ThermoFisher) containing 0.9mM MgCl₂, 0.492mM CaCl₂) or 10% formalin. Three cubes were put into 7 ml polystyrene bijoux tubes (Sterilin, Newport, UK) with 5ml fixative and incubated overnight at 4°C on a roller. After fixation, cubes were washed with PBS containing MgCl₂ and CaCl₂, twice for 10 minutes, once for 2 hours, then overnight at 4°C on a roller to remove fixative. Samples
were dehydrated through graded alcohols to xylene, embedded in paraffin wax and sectioned (5µm; C&C Laboratory Services, London, UK). After rehydration, sections were washed with water and stained with haematoxylin for 5 minutes then rinsed with water twice for 5 minutes each. Sections were stained with eosin for 2 minutes and washed with water for 3 minutes. Stained sections were dehydrated through graded ethanol (70–100%v/v), washed twice with xylene and mounted (Mounting medium; Dako, Carpinteria, US). H&E staining was adopted for routine morphological assessment (Chapter 2, Table 2.1). Images were captured using an Axiophot microscope (Zeiss, Cambridge, UK) and the Trio multispectral imaging system (Caliper, Runcorn, UK).

4.2.5 Transmission electron microscopy (TEM) of decellularised tissue cubes

Decellularised cubes were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in PBS without Mg/Ca (Electron Microscopy Unit, UCL Medical School, Royal Free Campus) for at least 12 hours at 4°C. After fixation, cubes were washed 3 times for 10 minutes each in PBS without Mg/Ca (Oxoid, Basingstoke, UK). Samples were post-fixed in 1% osmium tetroxide solution in PBS without Mg/Ca for 1 hour at 4°C and washed in water 3 times, 15 minutes each. Samples were dehydrated through graded ethanol (30%, 50%, and 70%) for 10 minutes each followed by 90% and 100% ethanol for 2 x 10 minutes, each. LEMIX resin (TAAB Laboratories) was warmed to room temperature and diluted to 50% resin solution with 100% ethanol. Samples were incubated in 50% resin solution for 12 hours at room temperature and in 100% resin for a further 6 hours at room temperature then placed in fresh resin in labelled embedding moulds and polymerised at 70°C for 12 hours. Sections (70nM) were cut on an Ultracut S (Leica, Milton Keynes, UK). The sections were placed on grids and stained with 2% aqueous uranyl acetate solution (to stain the phosphate and amino groups) for 4 minutes and lead citrate solution (to stain hydroxyl groups and osmium reacted areas) for 3 minutes followed by 5 washes in water. Grids were dried with filter paper and examined in a JEOL 1200–EX microscope using AMT V600 software (Deben UK Limited, Woolpit, UK). The processing, staining
and examination of sections of decellularised cubes were done by Ms Alejandra Carbajal (Electron Microscopy Unit, UCL Medical School, Royal Free Campus).

### 4.2.6 Sterility of decellularised scaffolds

Freshly decellularised cubes were placed in Dulbecco’s Modified Eagle’s medium (DMEM; Invitrogen) containing 10% foetal bovine serum (FBS; Sera Laboratories International, West Sussex, UK) and 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide. Cubes were then fixed and processed for H&E staining (Chapter 2, Section 2.3).

### 4.2.7 Proteomics of decellularised ECM

Proteomic analyses were performed as described in Chapter 2, Section 2.5. Six cortical tissue cubes from each of 6 different kidneys were decellularised. Decellularised scaffolds and intact tissue cubes (snap-frozen on receipt) from the 6 kidneys were enriched for cellular and ECM fractions, to enhance ECM protein identification, before being analysed by LC-MS/MS. Data from ECM and cellular fractions of each sample (intact kidneys and decellularised scaffolds), were merged together to compare all proteins identified in the decellularised scaffold with those of intact tissue. For positive identification, proteins had to have at least 2 spectral counts in each sample and to be present in 4 out of 6 samples in either decellularised or intact tissue samples (unlike the studies in the previous chapter (Chapter 3) in which the protein identification criteria was 2 out of 6 samples to allow comparison with previous published data (139)). Three ECM protein databases were selected to compare the coverage of matrix protein in different databases and the protein list was matched with each of the databases separately to generate an ECM protein list for further comparison between intact kidney and decellularised scaffold. The database for Human Matrisome Project (HMDB) (2) (http://matrisomeproject.mit.edu/), the GOTERM: extracellular matrix organization GO biological process annotation chart (GOTERM_BP_DERICT recommended by Dr. Ruth Lovering, Bioinformatics and Gene Annotation course organizer (UCL); new GO
category provided in Oct 2016, which provides GO mappings directly annotated by the source database (no parent terms included) at https://david.ncifcrf.gov/content.jsp?file=release.html and the UniProt database subcellular location: extracellular, were used for ECM protein identification.

4.2.8 Statistical analyses

The data are assumed to be normally distributed. The results of residual SDS concentration and residual DNA concentration were analysed using an unpaired T-test and mean ± SD was presented (Prism 7, Graphpad, San Diego, US), and the p<0.05 was considered significant.

4.3 Results

4.3.1 Optimisation of decellularisation of human kidney cortex cubes

A number of protocols have used water for the first stage of decellularisation (196, 223, 234). In order to assess how much decellularisation could be achieved with water alone, 5x5x5mm kidney cubes were decellularised using only water. In the first decellularisation experiment (Protocol for Decellularisation (PD)1, Figure 4.2) cubes were washed in water 12 times for 1 hour each, at room temperature with gentle agitation. The second experiment (PD2, Figure 4.2) used 6 x 1 hour water washes. Cubes were collected every 3 washes and fixed for H&E staining to assess cellularity. The centre of the cube was accessed from serial sections, since this is the region that is likely to be the most difficult to decellularise. With progressive washing, the wash solution became clearer, suggesting progressive removal of cellular material from the tissue. At the same time, with increasing numbers of washes the tissue cubes became more transparent. However, H&E staining of the cubes, sampled after both 6 (PD2) and 12 washes (PD1) showed incomplete decellularisation, with residual cellular material observed at the centre and edges of the cubes, i.e. cells still present within tubules, vessel walls and glomeruli (Figure 4.2B, D, F compared to C, E, G). The results also showed no differences in residual cellular components after 6 or 12 washes, suggesting the
maximum decellularisation using water only is achieved after 6 x 1 hour washes. Therefore, for subsequent protocols 6 x 1 hour washes with water were used which had the additional advantage of reducing the length of the protocol.

Figure 4.2 Decellularisation of human kidney cortex cubes using water. PD1 (12 x 1 hour washes) and PD2 (6 x 1 hour washes): Schematic representation of the decellularisation protocols indicating the different steps. A-C: Representative images of H&E stained sections at the end of the corresponding experiment, n=1 with 3 scaffolds/condition from the same kidney, ≥10 images/scaffold were examined. A: Intact kidney used for PD1 and PD2. B: Decellularisation after PD1. C: Decellularisation after PD2. D: Centre of cubes after PD1. E: Centre of cubes after PD2. F: Edge of cubes after PD1. G: Edge of cubes after PD2. Edge of the cubes and decellularised area are delineated by black lines. g: Glomeruli. Magnification: A-C: 10x; D, E, F, H: 20x.
To improve the extent of decellularisation achieved with water alone, different combinations of detergents were selected based on the successful published decellularisation achieved in whole kidney perfusion (196, 218, 233, 234): (i) 1% Triton X-100, (ii) 1% SDS, and (iii) 1% SDS with 1% Triton X-100 (Figure 4.3, PD3 and PD4). Cubes were washed 6 x 1 hour in water then incubated in detergents for 3 hours (all 3 detergent conditions) with no change of solution and shaking at room temperature (PD3, Figure 4.3). However, tissue decellularisation was still incomplete (of the 3 conditions, 1% SDS provided the most extensive decellularisation) (Figure 4.3 B-D). Cubes were incubated for a further 6 hours in detergent with shaking but no changes of solution (9 hours in total) (Figure 4.3, PD4). SDS (1%) treatment produced completely transparent cubes. In contrast, qualitative visual assessment showed the cubes subjected to other detergent treatments had a dark brownish region in the middle of the cubes suggesting incomplete decellularisation, which was confirmed by H&E staining (Figure 4.3E, F and G). Complete decellularisation was achieved with 1% SDS alone compared with 1% Triton X-100 or 1% SDS with 1% Triton X-100 (Figure 4.3F compared to E and G), and thus was used in further protocol development.

Since the aim of these experiments was to create decellularised tissue scaffolds for re-seeding cells (Chapter 5) it was important to remove any residual detergent. To this end, an overnight water washing (8 hours) was added after detergent treatment with no change of solution (Figure 4.4, PD5), and shaking at room temperature to remove residual SDS. To reduce the exposure of tissue samples to detergent and total protocol time, for the next protocol, the volume of the tissue cubes was reduced by half (5x5x2.5mm) and the time for each step reduced by half (Figure 4.4, PD6-7). Histological analysis showed that the same extent of decellularisation was achieved in PD5 and PD6 (Figure 4.4B compared to C). PD6 and PD7 used the same protocol, but during the last water wash in PD7 solution samples (500µl) were collected after 30 minutes, 1, 2, 3 and 4 hours) and SDS concentration measured using methylene blue (Figure 4.4D). There was no further change in SDS concentration in the wash solution in 1, 2, 3 and 4 hours.
Figure 4.3 Effect of different detergents on tissue decellularisation. PD3 and PD4: Schematic representation of the decellularisation protocols indicating the different steps. n=1 with 3 scaffolds/condition the same kidney, ≥10 images/scaffold were examined. A-G: Representative images of H&E stained sections at the end of the corresponding experiment. A: Intact kidney. PD3: B: Decellularisation with 1% Triton X-100; C: Decellularisation with 1% SDS. D: 1% SDS and 1% Triton X-100. PD4: E: 1% Triton X-100; F: 1% SDS; G: 1% SDS and 1% Triton X-100. g: Glomeruli. Magnification: 10x.
Figure 4.4 Optimisation of decellularisation and quantitation of residual detergent. PD5, 6 and 7: Schematic representation of the decellularisation protocols indicating the different steps. A-C: Representative images of H&E stained sections at the end of the corresponding experiment, n=1 with 3 scaffolds/condition the same kidney, ≥10 images/scaffold were examined. A: Intact kidney. B: PD5. C: PD6 and7. D: Measurement of residual SDS concentration at different time-points during the final 4 hours of water treatment in PD7, n=1. g: Glomeruli. Magnification: 10x.

compared with 30 minutes (Figure 4.4D) suggesting that after 30 minutes of water washing an equilibrium is reached and no further SDS can be eluted from the scaffold. This suggested that increasing the number of washes rather than the extended time of washing, might be beneficial.

To determine whether repeated washes increased SDS removal, the number and duration of post-SDS water washes were changed to 3 x 20 minutes (Figure 4.5, PD8). The SDS concentration at the end of each 20-minute wash was measured (Figure 4.5D), this showed that several changes of solution during this step markedly enhanced SDS removal. In some cases, a slight increase in the size of the cubes during water washes was observed presumably due to the absorption of water into the matrix. However, when
the cubes were transferred into SDS solutions, the original size was restored. It was also noted that with multiple changes of alternating detergent and water, cubes became increasingly transparent. Based on this observation, the protocol was modified to reduce the initial water washes to 20 minutes each and include alternating SDS (1 hour) and water (20 minutes) repeated 3 times followed by 4 x 20 minutes water washes to reduce the overall protocol time (Figure 4.5, PD9). The efficiency of decellularisation of the revised protocol was confirmed by H&E staining of the cubes (Figure 4.5B compared to C) which suggests that alternating cell lysis (detergent treatment) and debris removal (water washes) is as efficient as long detergent treatment for decellularisation. Residual DNA concentration was measured (Figure 4.5, PD8 and 9) to assess the removal of cellular components during decellularisation (Figure 4.5E, G). However, DNA quantitation showed only 50% DNA was removed.

Figure 4.5 Decellularisation and quantitation of residual detergent and DNA. PD8 and 9: Schematic representation of the decellularisation protocols indicating the different steps. A-C: Representative images of H&E stained sections at the end of the corresponding experiment, n=1 with 3 scaffolds/condition the same kidney, ≥10 images/scaffold were examined. A: Intact kidney. B: PD8. C: PD9. g: Glomeruli. Magnification: 10x. Residual SDS concentration in water washes (n=1): D: PD8. F: PD9. Residual DNA concentration after decellularisation (n=1): E: PD8. G: PD9. (The residual DNA level was measured using 3 scaffolds from n=1 experiment). Error bars: Mean + SD.

A recent liver decellularisation study showed that DNase treatment was required for complete DNA/cell removal (250) hence a DNase treatment step with preceded by
PBS washes (reaction buffer, 1M NaCl) was added to the end of the protocol to remove the residual DNA in the decellularised tissue (Figure 4.5, PD10). Surprisingly, although the same protocol as used in PD9 was applied, incomplete decellularisation was observed in PD10, with cytoplasmic proteins and DNA still present in the cubes (Figure 4.6A, B, and D). The level of residual DNA in PD10 was similar to the residual DNA level in PD8 in which there was no DNase treatment (Figure 4.6D compare to Figure 4.5E). One explanation might be that the DNase used was not active. In the next procedure (PD11), 2.4mM MgCl₂ and 0.5mM CaCl₂ were added to the reaction buffer (398) (Figure 4.6, PD11). After detergent and water treatments, cubes were incubated with DNase for (PD11). The results showed that maximum removal of residual DNA was achieved with

![Figure 4.6 Decellularisation and removal of residual DNA by DNase treatment.](image)

PD10 and 11: Schematic representation of the decellularisation protocols indicating the different steps. A-C: Representative images of H&E stained sections at the end of the corresponding experiment, n=1 with 3 scaffolds/condition the same kidney, ≥10 images/scaffold were examined. A: Intact kidney. B: PD10. C: PD11. g: Glomeruli. Magnification: x10. D: Measurement of residual DNA concentration after 3 hours DNase treatment in PD10. E: Residual DNA test after different DNase treatment times, PD11, n=1 (The residual DNA level was measured using 3 scaffolds from 1 experiment). Error bars: Mean + SD.
30 minutes, 1 or 3 hours at 37°C rolling at 30 rpm followed by 3 x 20-minute water washes.

1 hour DNase treatment followed by water washes (Figure 4.6E). Incubation in DNase solution, tended to make the scaffolds slightly “sticky.”

The incomplete decellularisation observed in PD10 and 11 (Figure 4.6B, C) might be due to the reduction in duration of detergent and washes (PD8 to PD9). Therefore, the initial water wash step was increased to 7 x 30 minutes and the SDS/water treatment repeated an extra time with water treatment extended to 30 minutes (Figure 4.7, PD12). In addition, the DNase incubation step was extended to 2 hours to ensure the removal of residual DNA from the decellularised scaffold. Using this new protocol (PD12), the removal of cellular components was confirmed by H&E (Figure 4.7B), and the residual DNA content was reduced to 35 ng/mg (Figure 4.7D) which is consistent with other kidney decellularisation protocols possibly suggesting that there is a small amount of DNA remains trapped in the matrix and cannot be removed (196, 216, 218, 220-223, 234). This protocol was repeated (Figure 4.7, PD13) and complete decellularisation was confirmed by H&E staining (Figure 4.7C, E) and measurement of residual DNA concentration (Figure 4.7D and E). After decellularisation, the colour of kidney cubes changed from dark brown to complete white/transparent (Figure 4.7F) and the scaffolds were slightly harder to handle with more jelly-like consistency. This final decellularisation protocol (PD13) was used for all further experiments. In all cases, H&E of decellularised cubes was assessed at the end of the protocol to confirm removal of cellular material.

**4.3.2 Sterility of scaffolds for in vitro use**

To use the decellularised cubes as scaffolds in which to seed cells, the cubes need to be sterile. Therefore, tissue cubes were decellularised according to PD13 using sterile solutions and aseptic conditions. The cubes were placed in individual wells of a 12-well plate in culture medium (for HK-2 cells) with 10% FBS and incubated at 37°C in a humidified atmosphere of 5% CO₂. The cubes were monitored by phase contrast microscopy over 2 weeks for qualitative signs of contamination. No contamination was observed over this period, the cubes remained completely transparent and the medium
Figure 4.7 Confirmation of the optimised decellularisation protocol. PD12 and 13: Schematic representation of the decellularisation protocols indicating the different steps. A-C: Representative images of H&E stained sections at the end of the corresponding experiment, n=1 with 3 scaffolds/condition the same kidney, ≥10 images/scaffold were examined. A: Intact kidney. B: PD12. C: PD13. g: Glomeruli. Magnification: 10x. Measurement of residual DNA concentration (n=1, The residual DNA level was measured using 3 scaffolds from 1 experiment), D: PD12, E: PD13. F: Representative pictures of kidney cubes before (left) and after (right) decellularisation. Error bars: Mean + SD. Absence of contamination was confirmed by H&E staining of the fixed and embedded cubes.

4.3.3 Ultrastructure of decellularised tissue

As shown in Figure 4.7, decellularisation removed the cellular elements of the kidney cortex as assessed by H&E staining. Tissue ultrastructure following decellularisation was examined by TEM. Different ECM compartments could be identified (Figure 4.8A) within glomeruli including the mesangial matrix (Figure 4.8B, C), glomerular basement membrane (Figure 4.8B, C), and Bowman’s capsule (Figure 4.8A, B, C). Distinct basement membranes were apparent around lumens within the tubulointerstitium and bundles of collagen fibres were present in the interstitium (Figure
Electron dense areas were observed along the Bowman’s capsule, GBM and TBM but have not yet been characterised (Figure 4.8B-E).

Figure 4.8 TEM of decellularised kidney cortex. Decellularised kidney cortical cubes generated by PD13. A: Low power scan of the tissue (circle indicates a single glomerulus (g); red quadrangles (B-E) indicate the area of where high magnification images were taken; black lines are the supporting grid), B-E: High magnification images of the rectangles outlined in red in A; *: Electron dense areas; #: Collagen fibres. BC: Bowman’s capsule. GBM: Glomerular basement membrane. MM: Mesangial matrix. TBM: Tubular basement membrane. N=1 experiment with 3 scaffolds/condition, ≥10 images/scaffold were examined.

4.3.4 Proteomics of ECM of intact and decellularised human kidney cortex

In order to determine the composition of decellularised scaffold and how decellularisation affects the ECM, LC-MS/MS was used to analyse the decellularised scaffolds and the intact kidney tissue (6 human kidneys aged >60 years old). In this chapter, different criteria were applied to analyse MS result compared with the previous chapter (details see Section 4.2.7). In Chapter 3, the criteria used were applied in order to be consistent with published glomerular ECM study (139). In this chapter, new categories from HMDB (published by Naba et al., 2016) (2) including collagens, glycoproteins, proteoglycan, ECM regulator, ECM-affiliated protein and secreted factors, were adopted. After merging ECM and cellular fractions for each sample, a total of 478 proteins were identified (Appendix Table 4.1) in the decellularised samples compared
with 1082 proteins in intact tissue (Appendix Table 4.2). Three different databases, HMDB, UniProt, and GOTERM: extracellular matrix organization (GOTERM_BP_DIRECT) were used to analyse the proteins detected in the decellularised scaffolds vs intact tissue samples. Although the HMDB is most commonly used to identified matrix proteins, the UniProt database did provide information on protein localisation and GOTERM provided information of proteins which are involved in ECM organisation. The commonality or differences of these three databases in identifying matrix proteins in decellularised samples are evaluated in the Discussion (Chapter 4, Section 4.4). The HMDB, identified 63 ECM proteins in the decellularised samples and 91 ECM proteins in intact tissue samples (Figure 4.9). Of these ECM proteins, 58 were

Figure 4.9 Comparison of ECM proteins identified by the HMDB in intact and decellularised human kidney cortex. The number of proteins in each section is indicated in bold at the bottom of each group. Blue: Only identified in intact tissue. Green: Identified in both intact and decellularised samples. Brown: Only identified in decellularised samples.
common to both intact and decellularised tissue, including 17 collagens (COL6A3, COL6A2, COL6A1, COL5A2, COL5A1, COL4A6, COL4A5, COL4A4, COL4A3, COL4A2, COL12A1, COL14A1, COL4A1, COL18A1, COL1A1, COL1A2 and COL3A1), 23 ECM glycoproteins (AGRN, VWA1, VTN, TINAGL1, TINAG, THSD4, TGFBI, POSTN, NPNT, NID2, NID1, LAMC1, LAMB2, LAMB1, LAMA5, FRAS1, FN1, FGG, FGB, FGA, FBN1, EMILIN1 and DPT), 4 proteoglycans (LUM, HSPG2, BGN and ASPN), 6 ECM regulators (TIMP3, AMBP, TGM2, SERPINA1, F9 and CTSD), 7 ECM-affiliated proteins (FREM2, CLEC18A, ANXA11, ANXA2, ANXA4, ANXA6 and ANXA7) and 1 secreted factor (INHBE). Most of the core matrix proteins (44 out of 57 proteins) including the 17 collagens, 23 ECM glycoproteins and 4 proteoglycans, were retained after decellularisation (Figure 4.9). Of the 33 ECM proteins only identified in intact kidney, there were 12 ECM regulators (ADAM10, CTSA, CTSB, CTSG, CTSH, ITIH5, SERPINA3, SERPINA5, SERPINB1, SERPINB6, SERPINC1 and SERPINH1), 8 ECM-affiliated proteins (ANXA1, ANXA5, FREM1, HPX, LGALS1, LGALS3, LMAN1 and PLXNB2), 8 ECM glycoproteins (AEBP1, LAMA1, LAMA4, MATN2, MFGE8, SBSPON, THBS1 and TNC), 4 proteoglycans (DCN, OGN, PRELP and VCAN) and 1 collagen (COL15A1). The 5 proteins only identified in decellularised tissue were COL16A1, COL21A1, and FBLN2, HRNR and S100A11. The UNIPROT database identified 50 extracellular proteins in decellularised tissue and 66 proteins in intact tissue (Figure 4.10). Out of the 50 proteins identified in the decellularised scaffolds, 47 were shared with intact kidney (Figure 4.10). COL16A1, COL21A1 and FBLN2 were only evident in the decellularised samples. Nineteen proteins were only identified in intact tissue (ANXA1, C1QBP, COL15A1, CTSB, DCN, FREM1, GGH, HMGB1, LAMA1, LGALS1, OGN, PRELP, RPSA, SBSPON, SERPINA5, SERPINC1, SPRT1, TNC and VCAN). Matching the protein list with GOTERM: ECM organization (GOTERM_BP_DIRECT), identified 41 ECM proteins in decellularised tissue and 49 proteins in intact tissue (Figure 4.11) of which 39 were shared. COL16A1 and ITGA3 were only detected in decellularised tissue.
while 10 proteins were only identified in intact kidney (BSG, DCN, HSD17B12, ITGA6, LAMA1, LAMA4, THBS1, TNC, TTR and VCAN).

**Figure 4.10** Comparison of ECM proteins identified by the UniProt Database in intact and decellularised human kidney cortex.
The number of proteins in each section is indicated in bold at the bottom of each group. Blue: Only identified in intact tissue. Green: Identified in both intact and decellularised samples. Brown: Only identified in decellularised samples.

**4.4 Discussion**

The overall goal of this thesis was to characterize the normal human kidney cortical ECM and to explore how the complex matrix regulates the behaviours of different renal cell types within this tissue compartment. The study focussed on the cortex since this is the region affected during ageing of the kidney (see Chapter 3) and it is the changes in the cortical tubulointerstitium that are relevant to the progressive fibrosis in
CKD (11). One approach to understanding how the complex in vivo ECM regulates the biological functions of different cells in the kidney cortex, is to seed cells into a decellularised ECM scaffold in which tissue architecture and ECM compartmentalisation are retained. To achieve this goal, in this chapter, an optimised decellularisation protocol was developed to generate sterile human kidney cortex ECM scaffolds for in vitro studies. A tissue cube-based decellularisation approach was adopted as the supply of fresh human kidney tissue is limited and the whole kidney is not always available for decellularisation.

Previously, most of the studies involving decellularisation have used whole kidney perfusion (193, 196, 216-221, 223, 233, 234, 399). Depending on the kidney size...
decellularisation of whole organs takes from 10 - 30 hours for rat (196, 216-219); 2 - 14 days for pig (220, 221, 223) and human kidneys (233, 234). More recent studies have used kidney slices rather than the whole organs to maximise the use of tissue (220, 222). For example, slices of pig kidney (211, 222) were decellularised within 14 days. At the time the current study was started there were no reports of decellularisation of human kidney tissue slices/cubes although recently 2 studies have been published, in 2016 Nagao et al. (168) reported decellularisation of 1cm cubes after 10 days (a total of 5 days continuous exposure to 1% SDS) and in 2018 Bombelli et al. (235) reported decellularisation of 2mm cubes after 28 hours (a total of ~12 hours of continuous exposure to 1% SDS with 1 hour 0.02% Trypsin, 2 hours 2% Tween-20, and 3 hours 4% sodium deoxycholate) (Figure 4.12).

Figure 4.12 Comparison of protocols used in different cube/slice-based decellularisation of the human kidney.

Both protocols included substantially longer exposure to detergents than the protocol developed in our study. The shorter exposure would likely be beneficial in retaining matrix proteins, however, it is not possible to directly compare the matrix composition in the different studies as Nagao and colleagues only report 26 proteins from their proteomic analysis and while no proteomic analysis was reported in the study by Bombelli et al. (168, 235). In our protocol, aseptic technique and sterile solutions were used for all steps and decellularised scaffolds were tested for contamination by incubation in culture medium for 2 weeks at 37°. In contrast, following decellularisation
Bombelli et al. used a long sterilisation procedure including a total of 12.5 hours of antibiotic treatment and PBS washes (235). Our protocol produced fresh scaffolds in ~14 hours with no sterilisation steps required which means the preparation of scaffolds is feasible as part of an experimental protocol. It provides a relatively fast production with no requirement for longer term storage of scaffolds which can potentially increase the risk of contamination (at 4° in 10% antibiotic solution (223)) or alter the scaffolds (by turning matrix into powder (168)). Thus, our study achieved efficient decellularisation with reduced exposure to detergents and produced sterile scaffolds within a practical time-frame with no requirement for storing scaffolds.

In developing a tissue cube-based decellularisation protocol, a number of variables were considered. The first was the size of the tissue samples. Considering published data of the size variation of the tissue slices or cubes used for decellularisation from coronal and transverse slices of pig and monkey kidney of 2mm thickness to 10mm cubes (211, 222, 232), median size of 5x5x5mm^3 was initially selected. However, this was subsequently reduced to 5x5x2.5mm^3 to improve decellularisation efficiency.

Other than the size of kidney cubes, the choice of the chemicals used for removing cellular components is key to effective decellularisation. Most decellularisation protocols start with incubation in PBS (to wash tissue) or water (to wash tissue and cell lysis) (193, 196, 216-221, 223, 233, 234). In our protocol initial water washes were adopted since this achieves both cell lysis (osmotic pressure) and removal of debris while a PBS washes only remove the cell debris and require an additional cell lysis step. Although published reports suggested that detergent treatment is necessary for effective decellularisation of large pieces of kidney (220, 233, 234) as the size of kidney tissue cubes in this study was small, the efficiency of decellularisation of water was initially tested. The complexity of the decellularisation protocol could be significantly reduced ie. no wash steps to reduce potential cytotoxicity of chemicals, low contamination risk with fewer solution changes, if the decellularisation could be achieved by using water only. However, although the use of water only did result in some decellularisation this was
incomplete (Figure 4.2) suggesting that more stringent conditions, such as detergent treatments, were required to achieve full decellularisation even of relatively small tissue cubes.

As reviewed in Chapter 1, Section 1.3.2, whole organ decellularisation protocols frequently use detergents. Each of the most common ionic and non-ionic detergents used in previous decellularisation protocols was tested on the human kidney cubes (1% Triton X-100 only, 1% SDS only and 1% Triton X-100 with 1% SDS) (193, 196, 217, 218, 233, 234). H&E staining has routinely been used to assess the level of decellularisation in a variety of protocols (193, 196, 211, 217, 218, 233, 234) and was adopted as a means to evaluate decellularisation efficiency in our studies. The data showed that 1% SDS resulted in the most efficient decellularisation. This result is consistent with a recent study showing that 1cm cubes of human kidney tissue could be decellularised using an SDS-based protocol (168). Wang and colleagues tested 5 different detergents and chemicals for decellularising whole pig kidney and also showed 1% SDS to be the most efficient (223). However, Choi et al. showed that although treatment with 1% SDS for 10-12 days removed all cellular components for pig kidney cubes, this prolonged treatment induced some ultrastructural damage (222); a finding confirmed by other investigators (220, 223). Therefore, it was considered important to minimise the duration of SDS treatment to better preserve the ECM. Despite the difference in size between rat and pig kidneys, in some cases whole rat kidney required a period of 30 hours for decellularisation (217) and pig kidneys could be decellularised in 2 days (223). However, in those publications, there did not appear to be any intermediate monitoring during decellularisation and decellularised tissue samples were only examined at the end of protocol making it impossible to determine whether these long incubation times were necessary (193, 211, 217, 218, 220-223, 234). This emphasises the potential advantage of qualitatively monitoring tissues throughout the protocol to minimise the time of exposure to detergents. Based on the study by O’Neill et al. (211), increasing the number of changes of solution in both detergent and water washes was found to enhance decellularisation and reduced
the duration of SDS treatment from 9 to 4 hours. Our final protocol used 4 x 1 hour
washes in 1% SDS (211). Interestingly this was consistent with a subsequent time-
course study in whole rat kidneys which demonstrated the feasibility of reducing the SDS
perfusion time from 24 to 4 hours with a commensurate improvement in ECM
ultrastructural preservation (216). In our protocol, the time of kidney cubes was exposed
to 1% SDS is significantly shorter than in the protocols published after the development
of our protocol which required incubation of human kidney cubes in 1% SDS for 5 days
(168) and overnight (235), respectively. It could be speculated that our protocol would
generate scaffolds with better ultrastructural preservation of ECM given the much shorter
SDS treatment time. Although He et al. showed successful decellularisation of whole rat
kidney with 0.125% SDS vs 1% SDS, with better preservation of growth factors in the
matrix (216), our study did not examine the effect of concentrations of SDS less than 1% on
human kidney decellularisation because this study was focused on reducing the time
of exposure to SDS. It is possible that reducing the SDS concentration might be
beneficial, but as our protocol provided efficient decellularisation and good preservation
of ECM components and structure with short time-frame. Therefore, additional
concentrations for detergent treatment were not tested in this study. As detergent is likely
to be toxic to cells seeded into decellularised cubes, additional steps to remove any
residual SDS were added to the protocol such that the concentration of SDS in the final
wash solutions was <0.001%.

Measurement of residual DNA (quantitation of DNA content or DAPI staining)
provides another way to determine the efficiency of removal of cells/cellular debris (196,
216, 218, 220-223, 234, 250). Some residual DNA was detected in the decellularised
scaffold (~200ng/mg) during the development of our protocol (Figure 4.5). When
reviewing the literature, it was noted that other investigators (220, 222, 223) used DNase
to remove residual DNA following decellularisation, thus a DNase step was added to our
protocol. The DNA content of the decellularised cubes was subsequently reduced 10-
fold compared with intact kidney samples (decellularised tissue 37±9.3ng/mg vs intact
tissue 352±63.7ng/mg). A similar reduction in DNA content was also observed in whole rat and pig kidney decellularisation (219, 223) and human slice-based decellularisation (235).

Many studies of tissue decellularisation have used H&E staining of paraffin-embedded sections to check for the removal of cells and assess general ECM structures (168, 211, 217, 218, 221-223, 232, 234, 235), however, light microscopy of H&E stained sections can only provide limited information on the decellularised matrix. Therefore, an increasing number of studies use electron microscopy (transmission/scanning) to assess the fine ultrastructure of the decellularised ECM (168, 196, 211, 217, 218, 220, 222, 223, 233, 234). In this study, TEM analysis of the human kidney cortex ECM using the optimised decellularisation protocol showed good preservation of glomerular ultrastructure (Bowman’s capsule, mesangial matrix, glomerular basement membrane) and the tubulointerstitium (basement membranes, interstitial collagen bundles and vessel walls). Similar ultrastructure had been reported by other investigators following kidney decellularisation (217-219). Bundles of collagen fibres were present in the decellularised human kidney cubes indicating SDS did not damage collagen fibres as had been suggested by Choi et al. (222). Several studies, including ours have reported electron dense areas along the basement membranes, however, these remain to be characterised (217-219).

Proteomics allows an unbiased evaluation of the protein content of the ECM and a means of assessing the potential differences in the ECM pre- and post-decellularisation as a result of the chemical removal of the cellular components and also the possibility of stripping some matrix constituents by the decellularisation process. There were 1082 proteins identified in the intact kidney cortex compared with a total of 478 proteins in the decellularised samples. This result also revealed that a similar complexity of our human kidney scaffolds to decellularised Rhesus monkey kidney slices in which 439 proteins were identified (232), although in that case transverse sections of the whole kidney were used compared to just the cortex. Unfortunately, the authors did not publish the details
of the proteins identified so it was not possible to determine to what extent the proteins obtained from human kidney ECM overlapped with those of the primate kidney. A recent proteomic analysis of decellularised human kidney cortical tissue cubes (168) reported only 26 proteins, although it was clear from the publication that other proteins were identified. However, the total number of proteins identified, and details of methodology used for protein identification were not reported and discussed so a direct comparison with our data was not possible. However, comparison of the list of proteins obtained in our study using HMDB showed that of the 26 proteins described 25 proteins were also identified in our study with the exception of elastin (168). Elastin is a principal component of aortic walls (400) and examination of H&E sections of 6 decellularised scaffolds in our study showed a maximum of 4 vessel profiles within each scaffold suggesting the contribution of elastin to the total ECM protein composition may be below the level of detection in LC-MS/MS. This was supported by the data in Chapter 3, on ECM from intact human kidney tissues, in which the spectral count of elastin was very low (2 to 7) in 4 out of 6 samples in the TI fraction and there was no elastin detected in the glomerular fraction, suggesting that there was very low amount of elastin in kidney cortical matrix. In 2018, another study decellularised whole human kidney and applied proteomics to analyse the ECM (162), and reported 92 proteins categorised into 6 groups including collagens, glycoproteins, proteoglycans, ECM-regulators, secreted factor and extracellular space (database used was not reported) (162). Comparing the list of proteins obtained by Leuning et al. (162) with our protein list (HMDB, 63 proteins), showed 43 proteins were detected in both studies. Matching the proteins identified by Leuning et al. (162) with HMDB, returned 71 proteins returned indicating that the choice of database used to identify matrix protein can affect the number of proteins identified. These results highlight the impact of using different databases which can make direct comparison of studies difficult. It is important that full lists of proteins detected should be published to allow comparison of matrices generated by different decellularisation methods.
Although using ECM protein-defining databases (commonly used in matrix protein studies) reduced the number of protein identified (from 478 proteins to 63 (HMDB), 50 (UniProt) and 41 (GOTERM)), in this study, three different databases were used to examine the commonality or difference in protein identification (Figure 4.12). The HMDB is the most commonly used database for matrix protein studies (139, 167), UniProt is a widely accepted platform for protein information, and GOTERM is often used for functional or pathway analysis of proteins. Among these 3 databases, there were 35 proteins, including 17 collagens, 11 ECM glycoproteins and 7 proteoglycans, identified in all three databases indicating a group of core matrix proteins included in different databases. Some differences in the proteins identified were also observed depending on the database used. For example, comparing proteins identified in the HMDB and UniProt, ATP5A1 (ATP synthase subunit alpha, mitochondrial) and CALR (Calreticulin, Ca\(^{2+}\)-Binding proteins produced by endoplasmic reticulum) appeared in the UniProt (these proteins were labelled as ‘extracellular space’ suggesting that these proteins had previously been identified in the extracellular space (401, 402)) but not the HMDB lists (Figure 4.13A). Comparing the HMDB and GOTERM lists, ITGA1 (integrin subunit α1), ITGA3 (integrin subunit α3) and ITGB1 (integrin subunit β1) were only identified in the GOTERM (Figure 4.13B). All these 5 proteins are intracellular rather than ECM proteins per se (403-408) highlighting the different criteria used to define matrix proteins in the different databases. Comparison of the proteins identified in UniProt and GOTERM showed the biggest differences between these two databases, 6 proteins in the GOTERM list were not identified in UniProt and 15 out of 50 proteins in the UniProt list were not found in GOTERM list (Figure 4.13C). These comparisons suggested the HMDB provides better overall coverage of ECM proteins compared with the other two databases, which is perhaps not surprising given that the HMDB is a specialised ECM-focused database. Similarly, comparison of three databases for identifying shared ECM proteins between intact tissue and decellularised samples, showed that most of shared ECM proteins identified by the UniProt and GOTERM were also detected by the HMDB.
Figure 4.13 Comparison of ECM proteins identified in HMDB, UniProt and GOTERM.

A: ECM proteins in HMDB compared with UniProt. B: ECM proteins in HMDB compared with GOTERM. C: ECM proteins in UniProt compared with GOTERM. The number of proteins in each section is given in bold at the bottom of each section.
Regardless of the database used there were a few proteins that were either not
detectable or only detectable in decellularised samples, which suggested that those
proteins not detected have been removed by decellularisation procedure while those
only detectable after decellularisation only reached levels of detection in the absence of
all the other proteins present in intact tissue samples.

In our study use of the HMDB to filter the 478 proteins identified in the
decellularised human kidney cortex samples, identified only 63 ECM proteins. However,
given that histology and DNA quantification confirmed complete removal of cellular
components, the proteins left in the decellularised scaffold must be matrix-related
proteins ie. an additional 415 proteins not included in the HMDB. Within the 478 proteins
identified, there were several large protein groups, including collagens, ATP synthases
and heat shock proteins. Not surprisingly collagens were the biggest group of proteins
identified in the decellularised samples and included 19 collagen subunits. The second
largest group was ATP synthases (12) which produce ATP from ADPs (409), these
enzymes are generally localised to mitochondria and plasma membranes (409, 410)
and produce extracellular ATP and regulate intracellular pH (410, 411). Several ATP
synthases such as ATP1A1 (412), ATP1B1 (413) and ATP5F1A (414) have been
identified in extracellular regions. However, the localisation and function of ATP
synthases in kidney ECM is unclear. There were also 8 heat shock proteins identified in
the decellularised scaffolds. HSP90 has been implicated in the response to injury in the
kidney. After ischemia, HSP90 levels is induced in rat kidney and an HSP90 inhibitor can
suppress ECM accumulation in the rat UUO model (415). In rat tubular necrosis, level of
HSP60 was significantly increase in the proximal tubule (416). Similarly increased
HSP90 also reported in the paediatric patient with acute kidney injury suggesting that
increased HSP60 level could be used as a biomarker for kidney injury (417). It has been
shown that HSP proteins can be translocated into the extracellular space and play
important roles in other organs, for example, extracellular HSP90 can promote cell
migration and wound healing in pig skin (418) and extracellular HSP60 can induce
inflammation in rat cardiomyocytes (419). However, the function of extracellular HSP proteins in the kidney remains to be explored. Overall, the data suggest that although HMDB is a widely used database, it may underestimate the complexity of matrix and that analysis of decellularised kidney ECM reveals an even more complex matrix than had been suggested from proteomic analysis of ECM enriched samples (Chapter 3).

Comparing ECM proteins from intact and decellularised kidney cortex identified in HMDB, there were 91 proteins in intact tissue of which 58 were shared with decellularised tissue and 33 were only in intact tissue. There were 63 in decellularised tissue of which 5 proteins were only identified in decellularised tissue. After decellularisation, a complex core of proteins was retained composed primarily of structural proteins. Fifty-eight proteins were identified in both intact and decellularised tissues, most of which are core matrix proteins including 17 collagens, 23 ECM glycoproteins and 4 proteoglycans. Collagens were the biggest group of shared proteins. Fibrillar collagens including collagen I, III and V were detected in both intact tissue and decellularised scaffolds (420). There were also some non-fibrillar collagens, such as collagens IV and VI. Collagen IV isoforms are important components of basement membranes and collagen VI isoforms are important mediators linking collagen IV isoforms in basement membranes and collagen I isoforms in the interstitial matrix (421).

Of the ECM glycoproteins, LAMs were the largest group and were the major non-collagen ECM proteins (380). All six LAMs identified in both intact and decellularised samples were also detected in the TI matrix (Chapter 3). Aligned with our study, LAMA5, LAMB1, LAMB2 and LAMC1, which were identified in both intact and decellularised scaffold, were all previously reported in the human glomerular matrix (380, 422). One of the ECM regulators identified in both intact and decellularised samples was TGM2, which can be located both inside and outside the cell (423). Extracellular TGM2 facilitates crosslinking of ECM proteins such as FN or collagen I, and integrin β1 and syndecan-4-related cell-matrix interaction (363-365, 424). Studies have shown that TGM2 contains a 42-kD gelatin-binding
domain which binds to FN with high affinity (425, 426), forming a heterocomplex (423) which may explain the presence of TGM2 in the decellularised matrix.

A number of ECM proteins were lost from decellularised samples compared to intact tissue including 13 core matrix proteins (1 collagen, 8 ECM glycoproteins and 4 proteoglycans). The only collagen absents in the decellularised samples was COL15A1. The structure of COL15A1 indicates that it can self-assemble into multimers and via its interconnection with the basement membrane, may act as ‘spring’ in the matrix. EM images showed that COL15A1 was only loosely associated with the basement membrane with only the C-terminal associated with large collagen fibres (427) which may explain the loss during the decellularisation procedure. In the adult human kidney, collagen XV has been shown to be located mostly in the basement membranes of capillaries in the renal cortex, and some tubules and in the mesangial (428, 429). Very low to undetectable levels of collagen XV were observed in the normal human kidney TI (Chapter 3, Section 3.3.2) but was increased in diabetic nephropathy (429). In terms of the likely impact of the loss of COL15A1 on cells reseeded onto these scaffolds it is possible the absence of this collagen from basement membranes and the glomerular matrix may affect the behaviour of tubular epithelial cells and podocytes. Both LAMA1 and LAMA4 are basement membrane protein subunits, present in Bowman’s capsule, and tubular and in blood vessel basement membranes, respectively (45, 430, 431). Moreover, other decellularisation studies of human kidneys also demonstrated that LAMA1 and LAMA4 were not detected by proteomics after decellularisation (162, 168). Although why LAM subunit α1 and α4 are lost from decellularised matrix is unclear, it is possible that LAMA1 and LAMA4 subunits remained bound to the cell surface via receptors such as α-dystroglycan-mediated cell binding (LAM α1 subunit), syndecans (-1/-2 for LAM α1 subunit and -2/-4 for LAM α4 subunit) and integrins (α6β1, α6β4, and α7β1 for LAM α1 subunit and α6β1 for LAM α4 subunit), and were lost when cells were disrupted (432). Data from conditional LAMA1 and LAMA4 knock out mice (433) suggest that loss of these proteins from decellularised scaffolds is likely to have limited impact of
the behaviour of cells subsequently seeded onto the scaffolds although there may be some effect on mesangial cells and glomerular endothelial cells.

Apart from the core matrix proteins, there were 20 ECM-associated proteins only present in intact tissue including 12 ECM regulators and 8 ECM-affiliated proteins. It was suggested by Naba et al. (2), that ECM-associated proteins are more soluble than collagens, proteoglycans and ECM glycoproteins (2), and thus may be more easily be removed by decellularisation procedures. ECM regulators were the largest group of proteins, were present in the intact tissue but were largely absent from the decellularised matrix. Two families, the serpins and cathepsins, were identified as ECM regulators in the HMDB which suggested they may not be strongly integrated into the matrix and therefore vulnerable to removal by decellularisation procedures. Serpins are a superfamily of protease inhibitors (434). SERPINB1 and SERPINB6 are inhibitors of cathepsin G, but their localisation is not well defined (435, 436). The cathepsins (CTS) are large proteases initially found in lysosomes (279). Five CTS were detected in the intact kidney cortex (4) and decellularised samples (1), which these could be classified into three subfamilies: serine (cathepsin A and G), cysteine (cathepsin B and H) and aspartate (cathepsin D) proteases. Although the majority of members of these two ECM regulator families were only present in intact tissue, there were two proteins, SERPINA1 (serpin A1) and CTSD (cathepsin D) which were found in both intact tissue and decellularised samples. It was not clear why these two proteins withstand the decellularisation procedure while most of the other family members are removed, but may reflect stronger binding affinity to the matrix. Other studies confirmed the presence of SERPINA1 and CTSD in the glomerular matrix (139, 167). Among the ECM-affiliated proteins, there was a large group of annexins, the majority of which were present in both intact and decellularised samples and were reported in extracellular space and linked with apoptosis and inflammation (437).

Interestingly, there were 5 proteins that appeared to be only present in the decellularised ECM including 2 ECM-associated proteins (the secreted factors, S100A11, HRNR), 2 collagens (COL16A1, COL21A1) and 1 ECM glycoprotein (FBLN2). The low
spectral count for all these proteins may suggest they are only expressed at low levels in the kidney and as such were not detectable in the intact tissue where they were masked by the more abundant proteins such as collagens and LAMs. COL16A1 and COL21A1 were only apparent in the decellularised scaffolds both of which are less common non-structural collagens with only low mRNA expression in human kidney (438). Our study provides the first evidence of COL16A1 in the glomerular matrix (Chapter 3). There is currently no information on the function of COL16A1 in kidney, however, COL16A1 may potentially mediate integrin α1β1- and α2β1-related cell attachment and other changes in cell morphology (439). COL21A1 mRNA expression had been previously reported in the human kidney (440) and our study confirmed the presence of COL21A1 protein in the ECM, however, the function of COL21A1 in the kidney cortex ECM remains to be established. HRNR, S100A11 and FBLN2 were also only identified in decellularised scaffolds. Although HRNR is a secreted protein, it has been identified in the matrix of human breast tissue (441). In Chapter 3, HRNR was identified in both human kidney glomerular and TI matrix fractions although only at a low level. This may suggest that the apparent absence of HRNR in intact tissue in this study might be due to analysis of total tissue without enrichment of the tissue compartments. Alternatively, in the decellularised scaffold the removal of all cellular components may have unmasked HRNR protein, however, the spectral count was still low (2-7 counts in 5 out of 6 samples). S100A11 was detected in the matrix of both mouse fibrotic lung and mouse lung tumour matrix using proteomics and immunohistochemistry (442), suggesting the presence of S100A11 in ECMs. Analysis of the cortical ECM (Chapter 3) showed S100A11 was only detected in TI matrix at low levels (2 and 3 counts in 2 out of 6 samples). Moreover, the spectral count of S100A11 (2-4 in 4 out of 6 samples) in the decellularised matrix was relatively low suggesting the detection of this protein was likely due to the relative enrichment by decellularisation. FBLN2 can bind to the N-terminal region of fibrillin-1 and FN via integrin αIIbβ3 (443-446), and in bovine glomeruli there was co-localization of FBLN2 and fibrillin (445) suggesting FBLN2 was likely to be in the ECM. However, even
using ECM-enrichment (Chapter 3), FBLN2 was not detected in either the human TI or G ECM. Thus, the amount of FBLN2 in intact tissue is likely below the detection limit but is revealed by the removal of all cellular components, a suggestion which was supported by the low abundance (2 to 3 spectral counts in 4 out of 6 decellularised samples). The detailed functions of HRNR, S100A11 and FBLN2 in the kidney ECM remain to be established.

Proteomics of decellularised scaffold also revealed a substantial number of proteins that were not identified as matrix proteins in any of the three databases examined. A total of 478 proteins was identified by Scaffold software whereas the HMDB identified 63 proteins, UniProt 50 proteins and GOTERM 41 proteins. As decellularisation successfully removed cellular components, all the proteins in the remaining ECM scaffold should be matrix or matrix-associated proteins suggesting that there were a large number of matrix-related proteins which had not been included e.g. in the HMDB. This highlighted a potential limitation of use of databases in that they only contain the proteins that contain reported matrix structures or domains can/may remodel or interact with the matrix. Potentially data from decellularisation studies should be included in the matrix databases to expand the repertoire of proteins classified as ECM/ECM-related. Based on the protein composition of the decellularised human kidney tissue, our study had identified a range of proteins not previously recognised as ECM proteins and suggested that the complexity of the ECM may be underestimated.

In addition to ECM proteins, non-protein components may also be removed during decellularisation. Although, as discussed in Chapter 3 Section 3.4, non-protein ECM components were not examined in this study, there are several decellularisation studies in which changes in non-protein components were examined (211, 216, 220, 223, 235). Alcian blue staining of decellularised human and pig kidney slices was used to confirm the preservation of acidic GAGs following decellularisation (211, 235). Several studies have used sulphated GAG (sGAG) assays to determine the sGAG content of the decellularised scaffold compared with native kidney (211, 216, 220, 223). The sGAG
content was reduced 38% when 0.5% SDS treatment was used for 8 hours in
decellularised whole rat kidney compared with the native kidney (measured by: weight
of sGAG content/ weight of tissue) (216). In addition, a similar reduction (~45%) of sGAG
content was reported by Wang et al. in decellularised whole pig kidney (using 1% SDS
for 18 hours) compared with the native kidney (223). Similar to the whole pig kidney, in
decellularised pig kidney slices, there was a 35-50% decrease in sGAG content following
decellularisation (using 1% SDS for more than 10 days or a combination of different
detergents for 7.5 hours) (211, 220). Based on the results of decellularisation of pig
kidney slices, it seems likely there may be some loss of sGAG content in our
decellularised human renal cortical cubes although our shorter detergent incubation (4
hours, 1% SDS) might minimise the loss of sGAG content.

In summary, a protocol had been developed to decellularise cubes of human
kidney cortex with complete removal of cellular elements and retention of ECM
ultrastructure and complex protein composition. The entire protocol took 13 hours to
complete, with exposure of tissue to detergent limited to 4 hours and meant that fresh
ECM scaffolds could be prepared rapidly. Moreover, the short protocol together with the
use of sterile solutions, aseptic conditions and careful technique throughout the process
eliminated the need for sterilisation (radiation or antibiotic treatment) of the ECM
scaffolds. One added advantage of our protocol over whole organ decellularisation is
that smaller samples of tissue can be used, thereby maximising use and sharing of
scarce organs. This also meant that it was possible to test matrix cubes from a number
of different individuals and increase the number of biological replicates studied. Using
this optimised protocol sterile scaffolds were generated for use in in vitro experiments to
examine the effects of ECM on renal cell localisation and differentiation (Chapter 5).
Chapter 5

Repopulation of human kidney ECM scaffolds with human renal cells
5.1 Introduction

After the characterisation of the human kidney cortex matrix (Chapter 3) and development of a decellularisation protocol for kidney cortex tissue cubes (Chapter 4), the next step was to use the decellularised scaffolds to study how the kidney matrix regulates renal cell behaviour. There have been studies examining the repopulation of decellularised scaffold ranging from whole organ repopulation to repopulation of smaller scaffolds (tissue cubes) (244). As reviewed in Chapter 1 and 4, perfusion-based techniques have been used to decellularise the whole kidney from various species including mice, rat, pig and human, and to re-seed the decellularised whole organ scaffold with stem cells, endothelial and epithelial cells (211, 219, 234, 244).

Other studies have generated scaffolds of kidney ECM from tissue cubes which have been used to examine cell repopulation in a variety of settings including implantation of scaffolds in vivo into the kidney, onto the chick chorioallantoic membrane and in vitro culture. Yu and colleagues excised one-third of rat kidney from below the renal pelvis and implanted decellularised rat kidney scaffold into this pole of the kidney (217). Eight weeks post-implantation, the entire ECM scaffold was repopulated with inflammatory cells, and scar tissue was formed within the scaffold. However, no renal-specific structures were observed, such as glomeruli and tubules (217). Using a similar approach, Choi et al. implanted decellularised porcine kidney matrix cubes into the rat kidney (222). Histological analysis showed that cells from adjacent kidney tissue migrated into ECM scaffold within 6 weeks and by 12 weeks some glomerular- and tubule-like structures formed along the boundary between host tissue and implanted scaffold (222). Together, these data suggest that decellularised scaffold can support the migration and growth of cells from the healthy kidney, however, why glomeruli- and tubule-like structures were formed in pig scaffold but not in rat scaffold is unclear though might relate to the host immune or injury response or time post-implantation. In addition, kidney ECM scaffolds have also been shown to support angiogenesis. When decellularised human kidney ECM cubes were placed on the chick chorioallantoic
membrane, significantly more blood vessels were observed in kidney scaffold compared to the control polystyrene cube (234).

Studies using decellularised scaffolds in vitro have included looking at the effect of kidney matrices on stem cells and differentiated cells (plated on the top of scaffold). Mouse kidney stem cells (mKSCs) seeded onto decellularised pig kidney ECM slices exhibited a star-like morphology but did not produce any defined structures in the cortex, such as tubule- or glomeruli-like structures, while in the medulla cells presented an elongated morphology and formed tubule-like structures (observed using IFL (DPAI and phalloidin)), however the changes in the expression of cell markers was not examined (211). These data suggest that the kidney ECM can support the growth of mouse stem cells and may provide regional-specific architectural and compositional clues (211). Similarly, decellularised monkey kidney slices were reported to support the growth of human embryonic stem cells (hESCs) and formed tubular-like structures with increased expression of some tubular markers (aminoacylase 1 and fatty acid-binding protein 1) on RNA level during 8 days of incubation (232). However, both studies did not provide any detailed characterisation of cell type-specific markers of stem cells on protein level before or after seeding. A study by Bombelli and colleagues showed that primary human adult renal stem cells (expressing PAX-2 (renal progenitor markers), CD7 (distal tubule marker), co-expressing N-cadherin (proximal tubule marker) and E-cadherin (general epithelial marker) and some co-expressing CK8.18 (epithelial markers) and vWf (endothelial markers)) seeded on to decellularised human kidney slices formed epithelial- or endothelial-like structures and expressed compartment-specific markers such as CD10 (proximal tubular marker), CK7 and CK8.18 in epithelial-like structures, vWf in endothelial-like structures. Interestingly, the cells that reached glomeruli expressed epithelial markers (CK8.18) rather than podocyte-specific (synaptopodin and nephrin) or endothelial-specific (vWf) markers (235). Taken together these studies, suggest that decellularised kidney cubes/slices from one species can support the attachment and growth of stem cells from another species and the ECM may provide
compositional or structural clues for cell behaviours. When looking at species-specificity, most studies used cells from only one different species onto kidney matrix from another species, for example, human cells on pig matrix (220, 222) and human cells on mouse matrix (224). Therefore, it is impossible to directly compare or conclude anything about species-specificity, however, one study might vaguely suggest that there is benefit of putting human stem cells on decellularised whole human kidney matrix than on rat kidney (162).

Other than stem cells, a number of studies have examined the response of differentiated cells (primary cells and cell lines) seeded onto kidney cubes from a variety of species (220, 222). Primary human renal cells (a mixture of human kidney cortical cell types) were seeded onto porcine kidney matrix scaffolds (222). Another study also used a porcine matrix to examine its effect on human renal cortical tubular epithelial cells (220). Both studies showed that differentiated human renal cells could attach and grow on/in the porcine kidney ECM using H&E and cell counting (220, 222). However, no data on structure formation or expression of cell-specific markers were reported which make it impossible to determine whether any renal structure was formed (220, 222). Rather than using decellularised kidney scaffolds directly, Nagao et al. used decellularised human kidney ECM components to create a hydrogel containing an artificial vascular network, and tested the effect of this matrix on two types of endothelial cell (human kidney peritubular microvascular endothelial cells (HKMECs) and human umbilical vein endothelial cells (HUVECs)). Both endothelial cells could repopulate the micro-vessel structures created in the hydrogel, suggesting that kidney matrix gel could support endothelial cell growth and provide cues for vessel formation (168).

However, there have been only two studies looking at the effect of decellularised human kidney matrix on differentiated human renal cells are quite limited (162, 168). Therefore, the aim of studies described in this chapter was to examine the seeding of different human renal cells onto decellularised human kidney ECM scaffolds. Published studies have generally used, epithelial, endothelial, stem cells and primary kidney cells
to repopulate decellularised kidney scaffolds from different species (244, 245), however, very few of them using adult human kidney cells. To-date only human endothelial cells have been seeded onto decellularised human kidney matrix. Other human renal cell types have not been tested on human kidney ECM scaffolds nor has anybody compared the behaviours of different cell types on the matrix from the same kidney in parallel. Therefore, three representative cell types from the glomeruli and tubulointerstitium were selected for study, namely podocytes (a key epithelial cell in the glomerulus which controls blood filtration), proximal tubular epithelial cells (the predominant epithelial cells type in the tubulointerstitium) and interstitial fibroblasts (a key cell type involved in maintaining normal kidney structure and which plays an important role in fibrosis in multiple kidney diseases). The use of kidney cubes in decellularisation allowed us to examine the effect of the same matrix on the different cell types. Two main methods were used for seeding cells into decellularised scaffolds: (i) surface seeding whereby cells were deposited on the surface of the scaffold (232) and (ii) injection of cells into the matrix using syringe needle (230). In order to determine which method could achieve better repopulation, both methods were adopted for comparison in this study. Following seeding, the biological response of the cells was assessed using cell proliferation, apoptosis and differentiation assays.

5.2 Materials and Methods

General materials and methods are described in Chapter 2.

5.2.1 Recellularisation of ECM scaffolds

Tissue cubes from 3 kidneys of similar age (61 to 69) (details see Chapter 2, Table 2.1) were used to created decellularised scaffold for recellularisation. The changes in these kidneys include occasional sclerosed glomeruli, occasional calcified tubules, some thickening of the vessel walls and occasional fibrotic foci which are all described
as consistent with the age of the kidney when their histological sections were assessed by renal pathologist blindly.

Decellularised scaffolds were transferred into individual wells of a 12-well plate (Corning) with cell type-specific growth medium (Chapter 2, Section 2.3) and incubated overnight at 37°C in a humidified atmosphere of 5% CO₂. The following day scaffolds were transferred into individual wells of a 96-well round-bottom plate (Corning) to maximise matrix attachment, 4x10⁵ cells were seeded onto the scaffold (for all cell types) in 50µl growth medium and incubated at 37°C for 2 hours to promote cell attachment (the cell range was tested from 1x10⁵ to 4x10⁵, similar to studies described by Poornejad et al., Choi et al. and Nakayama et al.) (220, 222, 232). 150µl growth medium was then added to each well and incubated at 33 or 37°C (depends on cell type, see next paragraph) overnight to facilitate cell attachment. Cell-seeded scaffolds were then transferred to individual wells of a 24-well plate (Corning) with 1ml growth medium. Fresh medium was changed every 3 days. Initially, cultures were maintained for 1 – 3 weeks to determine the effect of time on repopulation, 2-week incubation was used for subsequent experiments. At the end of the culture period, scaffolds were fixed in 4% PFA in PBS for 2 hours, washed with PBS overnight and processed for histology (Chapter 2, Section 2.4).

Due to the conditional immortalisation of ciPod and tsHKF cells, these cells proliferate and differentiate at different temperatures (proliferating at 33°C and differentiating at 37°C). Therefore, after seeding with 50µl growth medium the ciPod and tsHKF cells were incubated at 33°C (permissive temperature) for 2 hours after seeding and then 150µl growth medium was added to each well and incubated at 33°C overnight in order to promote the attachment to the matrix. For HK2 cells, were incubated at 37°C for 2 hours after seeding and then 150µl growth medium was added to each well and incubated at 37°C overnight. Cell-seeded scaffolds were then transferred to individual wells of a 24-well plate (Corning) with 1ml growth medium. After changing to 24-well plate, scaffolds seeded with HK2 cells were incubated at 37°C, with ciPod cells were
incubated at 33°C (permissive temperature), and tsHKF cells were incubated at 33°C (permissive temperature) and 37°C (non-permissive temperature).

5.2.2 Data analysis of IFL

Details of IFL are given in Chapter 2, section 2.3. Cell nuclei were counted from ≥20 images per condition using ImageJ (version 1.51j8), and positive cells were counted manually. The percentage of each image was calculated. The Data were assumed to be normally distributed and analysed by unpaired T-test using GraphPad Prism (version 7.03) to determine the statistical differences between two groups. The value of result was expressed as mean ± standard deviation. The value of probability < 0.05 were considered significant.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>The range of number of cells counted on glass</th>
<th>The range of number of cells counted on matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK2</td>
<td>4755 - 6469</td>
<td>5190 - 9007</td>
</tr>
<tr>
<td>ciPod</td>
<td>6219 - 8913</td>
<td>2952 - 4265</td>
</tr>
<tr>
<td>tsHKF</td>
<td>2819 - 9113</td>
<td>3764 - 5689</td>
</tr>
</tbody>
</table>

Table 5.1 Numbers of cells counted of each experimental group (n=3 experiments) for different cell types.

5.2.3 Confocal microscopy

A fully-motorised Leica SP8 laser scanning confocal microscope (Leica) equipped with hybrid detectors and hardware-based autofocus controlled by Leica Application Suite X (LASX, version 3.5.1.18963; Leica) with Lightning super-resolution

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Objective</td>
<td>40x oil</td>
</tr>
<tr>
<td>Diffraction at back aperture of objective</td>
<td>1.3</td>
</tr>
<tr>
<td>Diameter of confocal pinhole</td>
<td>1 (airy unit)</td>
</tr>
<tr>
<td>Zoom</td>
<td>1.28x</td>
</tr>
<tr>
<td>Scanning frequency</td>
<td>Bidirectional 700Hz</td>
</tr>
<tr>
<td>Line average</td>
<td>2</td>
</tr>
<tr>
<td>Hybrid gain</td>
<td>100%</td>
</tr>
<tr>
<td>405 laser line</td>
<td>2.7%</td>
</tr>
<tr>
<td>561 laser line</td>
<td>0.6%</td>
</tr>
<tr>
<td>Z-step</td>
<td>0.28µM</td>
</tr>
<tr>
<td>DAPI detection spectra</td>
<td>410-556nm</td>
</tr>
<tr>
<td>Cleaved caspase-3 detection spectra</td>
<td>566-728nm</td>
</tr>
<tr>
<td>Pixel resolution</td>
<td>5400 x 5400 277.94µM²</td>
</tr>
</tbody>
</table>

Table 5.2 Parameters for confocal imaging of cleaved caspase-3.
module and 2 channel sequential scan was used to take confocal images. Images were analysed by Leica Application Suite X, and positive cells (tsHKF) of cleaved caspase-3 were count manually in each image. The percentage of each image was calculated.

5.2.4 Picro Sirius Red staining

Histological sections were rehydrated stained with Weigert’s Haematoxylin (Merck Millipore) for 10 minutes, washed in running water for 5 minutes and rinsed briefly with 1% acidified alcohol (1% acetic acid in ethanol). Sections were then stained with Picrosirius red solution (0.1% (w/v) Sirius red in saturated picric acid (1.3% (v/v) picric acid in water)) for 1 hour, rinsed twice in 1% acidified alcohol, washed in ethanol 3 times for 5-10s, dehydrated in xylene and mounted with mounting medium (Dako). Images were captured using an Axioskop 2 mot plus microscope (Zeiss) and the multispectral imaging system (AxioCam MRc5, Zeiss). At least 10 random images across the cortex of each sample were examined under both bright field and polarised light and representative images selected.

5.3 Results

5.3.1 Human proximal tubular epithelial cells on decellularised human kidney cortex ECM

Firstly, surface plating and injection of cells were compared in parallel to determine the efficiency of repopulation. HK2 cells (4 x 10⁵ cells) were either seeded onto or injected into the scaffold using an insulin syringe (29G, BD) (Figure 5.1). Reseeded scaffolds were incubated for 1 (Figure 5.1A, C) or 2 weeks (Figure 5.1B, D) to determine whether there is a time-dependent effect on recellularisation. These times were selected based on previously reported studies of reseeding kidney matrix cubes (211, 229) and tested (Figure 5.2) to determine the time which the most efficient recellularisation is achieved. Histological analysis of scaffolds reseeded by injection (Figure 5.1B, D) showed that small areas of closed approximate tubule-like structures
repopulated with a monolayer of cells were found in a small area of the scaffold. The assumptions are that these areas correspond to the sites of injection. There was no evidence of extensive repopulation of the scaffold after 2 weeks. Cells plated on the surface of scaffold could repopulate more extensively (Figure 5.1A, C) compared with injection at one and two weeks after seeding. Histological results also showed that cell could cover the surface of scaffold and aligned some tubule-like structures deeper with the scaffold with a monolayer of cells (Figure 5.1A, C). Therefore, surface seeding was adopted for the rest of recellularisation procedure.

![Figure 5.1 Effect of surface seeding vs injection on HK2 cell repopulation of decellularised scaffolds.](image)

HK2 cells (4 x 10^5 cells/scaffold) were introduced by surface seeding (A, C) or injection (B, D) of the scaffold and incubated for 1 week (A, B) and 2 weeks (C, D). Scaffolds were fixed, sectioned and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=1 experiment with 3 scaffolds/condition. Representative images of 10 images/scaffold are shown.

A longer time-course of incubation time was also tested. HK2 cells (4 x 10^5/cube) were seeded onto the scaffolds and incubated for 1, 2 or 3 weeks (Figure 5.2). As
previously shown, after 1 week, repopulation of the ECM scaffold was observed (Figure 5.2A), but there was only very limited repopulation of the scaffold, close to the surface with some tubule-like structures aligned with a monolayer of cells. Qualitative comparison of reseeded scaffolds incubated for either 1- or 2-weeks incubation, showed more extensive repopulation with more tubular profiles populated (with cell monolayers) over a larger proportion (into a greater depth) of the scaffold at 2 weeks (Figure 5.2A, B). Comparison of reseeded scaffolds incubated for 2 week or 3 weeks, showed repopulation extended to a similar depth into the scaffold but much less number of tubule-like structures repopulated at 3 weeks. Therefore, re-seeded scaffolds incubated for 2 weeks showed more extensive repopulation with more of the surface area covered by cells than after 3 weeks incubation suggesting that over the time-course tested cell repopulation was maximal at 2 weeks.

Next, the effect of the cell number seeded on repopulation of the scaffold was tested. Scaffolds were seeded with $1 \times 10^5$, $2 \times 10^5$ or $4 \times 10^5$ cells (Figure 5.3). After 2 weeks, the most extensive repopulation was observed (with repopulation extended to the deepest level and more tubule-like structures) in scaffolds seeded with $4 \times 10^5$ HK2 cells (Figure 5.3). For all subsequent experiments, $4 \times 10^6$ cells were seeded on the surface of the scaffold and incubated for 2 weeks.
Figure 5.3 Effect of HK2 cell number on repopulation of scaffolds.
HK2 cells were seeded at $1 \times 10^5$ cells (A), $2 \times 10^5$ cells (B) and $4 \times 10^5$ cells (C) /scaffold and incubated for 2 weeks. Scaffolds were fixed, sectioned and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=1 experiment with 3 cubes/condition; Representative images of 10 images/scaffold are shown.

Figure 5.4 Comparison of the histology of intact kidney, decellularised ECM scaffolds and scaffolds repopulated with HK2 cells.
Normal human kidney (A-C). Decellularised scaffold (D-F). Decellularised scaffold seeded with HK2 cells ($4 \times 10^5$ cells/scaffold) and incubated for 2 weeks (G-I). Samples were fixed, sectioned and stained with H&E and PSR. H&E staining (A, D, G). Brightfield image of PSR staining (B, E, H). Polarised light image of PSR staining (C, F, I). Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=3 experiments using scaffolds from 3 different kidneys (3 scaffolds/condition within each experiment); Representative images of 20 images/scaffold are shown.
The effect of repopulated epithelial cells on the matrix was examined using H&E and PSR staining. When compared with normal kidney (Figure 5.4A-C), the decellularised scaffold shows a similar ECM arrangement and collagen distribution (Figure 5.4D-F). HK2 cells did not induce any qualitative changes in the appearance of the ECM and collagen distribution (Figure 5.4G-I).

To further analyse the effect of matrix on HK2 cells, expression of markers of cell proliferation (Ki67), apoptosis (cleaved caspase-3), epithelial cell type-specific differentiation (Lotus tetragonolobus lectin (LTL), AQP1 and Dolichos biflorus agglutinin (DBA)) and mesenchymal markers (vimentin and α-SMA) were compared in cells seeded on glass vs decellularised scaffold. Seeding cells on the scaffolds significantly reduced proliferation (Figure 5.5A-D, I) from 94.62±0.42% Ki-67 positive cells on glass to 74.5±1.77% on the matrix. In parallel, there was an increase in cell death with staining of cleaved caspase-3 increasing from 3.51±0.41% positive cells on glass to 9.6±0.57% on the matrix (Figure 5.5E-H, J).

Expression of cell type-specific markers was also compared in HK2 cells seeded on glass vs ECM. Cells were stained with LTL and AQP1 (proximal tubule markers) and DBA (collecting duct marker). LTL-positive cells increased from 30.75±1.02% on glass to 93.22±1.03% on matrix (Figure 5.6A-D, M). It should also be noticed that LTL staining was localised around the nuclei in cells plated on glass, and more extensive cytoplasmic staining was observed in cells seeded on the scaffold (Figure 5.6B, D). However, there was no polarity staining of LTL in either group (447). A significant increase in AQP1 expression was also observed from 1.24±0.28-% on glass to 22.42±1.45% on matrix (Figure 5.6E-H, N). The collecting duct marker, DBA, was only expressed at background levels on both substrates (Figure 5.6I-L, O). Then, cells were stained with mesenchymal cell markers, including vimentin and α-SMA. The majority of cells expressed vimentin were not modified by the substrate: 99.84±0.05% positive cells on glass, 99.04±0.26% on matrix (Figure 5.7A-D, I). There appeared to be very weak diffuse cytoplasmic
Figure 5.5 Effect of glass substrate vs decellularised scaffold on HK2 cell proliferation and apoptosis.

HK2 cells were seeded on glass coverslips or decellularised ECM scaffold (4 x 10^5 cells/scaffold) and incubated for 2 weeks. Samples were then fixed and processed for IFL. Ki67 expression on glass (A, B) vs decellularised scaffold (C, D). Cleaved Caspase-3 expression on glass (E, F) vs decellularised scaffold (G, H). Sections and cells on coverslips were incubated with Ki67 antibody (B, D), cleaved caspase-3 (F, H) and an IgG control (A, C, E, G) for antibody specificity (IgG was used at an equivalent concentration to the primary antibody), nuclei were stained with DAPI. Percent positive cells for Ki67 (I) and cleaved caspase-3 (J) cells were counted (>20 images/condition were quantified). Arrows indicate positive cells. The insert (bottom left corner) shows the high-power image of weak positive cells. Representative images are shown of 20 images/condition; N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds/condition within each experiment). A-H: Magnification: 20x. **** p<0.0001. Error bars: Mean + SD.

expression of α-SMA (no staining of stress fibre-incorporated α-SMA observed), which was also unaffected by the substrate: 99.89±0.05% positive cells on glass, 99.88±0.04% on matrix (Figure 5.7E-H, J). Overall, the results indicated that HK2 cells could successfully attach to and repopulate the scaffolds forming a single layer of cells lining the lumens of tubular profiles within the decellularised matrix. Seeding on the scaffolds reduced cell proliferation, increased apoptosis and enhanced expression of proximal tubular epithelial cell markers in the HK2. Although there was an increase in expression of differentiated epithelial cell markers the majority of cells on both substrates were positive for the two mesenchymal markers tested (all cells expressed at a low level) suggesting that the ECM did not support complete differentiation of the cells.
Figure 5.6 Effect of glass substrate vs decellularised scaffold on the expression of tubular epithelial cell-specific markers.

HK2 cells were seeded on glass coverslips or decellularised ECM scaffold (4 x 10^5 cells/scaffold and incubated for 2 weeks). Samples were then fixed and processed for IFL. LTL expression on glass (A, B) vs decellularised scaffold (C, D). AQP1 expression on glass (E, F) vs decellularised scaffold (G, H). DBA expression on glass (I, J) vs decellularised scaffold (K, L). Sections or coverslips were incubated with LTL (B, D), AQP1 antibody (F, H), DBA (J, L), diluent control for lectin specificity (A, C, I, K) and IgG control (E, G) (IgG was used at an equivalent concentration to the primary antibody), nuclei were stained with DAPI. Percent LTL positive (M), AQP1 positive (N) and DBA positive (O) cells on scaffold vs glass (>20 images/condition were quantified). Arrows indicate positive cells. The insert (bottom left corner) shows the high-power image of weak positive cells. The insert (bottom left corner) shows the high-power image of weak positive cells. N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds/condition within each experiment); representative images of 20 images/condition are shown. A-L: Magnification: 20x. ****: p<0.0001. Error bars: Mean + SD.
Figure 5.7 Effect of glass substrate vs decellularised scaffold on the expression of mesenchymal markers.
HK2 cells were seeded on glass coverslips or decellularised ECM scaffold (4 x 10^5 cells/scaffold and incubated for 2 weeks). Samples were then fixed and processed for IFL. Vimentin expression on glass (A, B) vs decellularised scaffold (C, D). α-SMA expression on glass (E, F) vs decellularised scaffold (G, H). Sections or coverslips were incubated with vimentin (B, D) and α-SMA (F, H) antibody, and IgG control (A, C, E, G) for antibody specificity (IgG was used at an equivalent concentration to the primary antibody), nuclei were stained with DAPI. Percentage of Vimentin positive (I) and α-SMA positive (J) cells on scaffold vs glass (>20 images/condition were quantified). Arrows indicate positive cells. The insert (bottom left corner) shows the high-power image of weak positive cells. N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds/condition within each experiment); representative images of 20 images/condition are shown. A-H: Magnification: 20x. ns: not significant, **: p<0.01. Error bars: Mean + SD.

5.3.2 Human interstitial fibroblasts on decellularised human kidney cortex ECM

Another important cell type in the tubulointerstitium is the interstitial fibroblast. Although sparse in the normal kidney cortex, these cells play a key role in diseases characterised by progressive scarring (90, 91, 270, 448). Initially, normal primary kidney fibroblasts isolated from human kidney were used to repopulate the scaffolds. As described for HK2 cells, the effect of surface seeding vs injection of fibroblasts (4 x 10^4 cells/scaffold) on repopulation of the scaffold was tested. Regardless of the seeding method or length of incubation (1 or 2 weeks), a very limited number of fibroblasts were observed in the scaffolds (Figure 5.8A-D). The effect of cell number was also tested, 4.125 x 10^4, 8.25 x 10^4 and 1.65 x 10^5 fibroblasts/scaffold were seeded on the surface of the scaffold and incubated for 2 weeks. The tested cell number was determined by the number of cells available from the primary cultures. Of note, normal kidney fibroblasts proliferate slowly in vitro. Again irrespective, of the starting cell number, only a few cells were observed in the scaffolds after 2 weeks of culture (Figure 5.9C).
Figure 5.8 Effect of surface seeding vs injection on primary human kidney fibroblast repopulation of decellularised scaffolds.
Fibroblasts (4 x 10^4 cells/scaffold) were seeded on the surface of the scaffolds (A, C) or injected into the scaffold (B, D) and incubated for 1 week (A, B) or 2 weeks (C, D). Scaffolds were fixed, sectioned and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=1 experiment with 3 scaffolds/condition, representative images of 10 images/scaffold are shown.

Figure 5.9 Effect of primary human kidney fibroblast number on repopulation of decellularised scaffolds.
Cells were seeded on the surface of the scaffold and incubated for 2 weeks. 4.125 x 10^4 cells/scaffold (A) 8.25 x 10^4 cells/scaffold (B) 1.65 x 10^5 cells/scaffold (C) and grown for 2 weeks. Scaffolds were fixed, processed for histology and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=1 experiment with 3 scaffold/condition. Representative images of 10 images/scaffold are shown.
To determine whether the apparent failure of fibroblasts to attach the scaffold was unique to renal fibroblasts and/or possibly reflect the limited in vitro proliferative capacity of primary fibroblast cultures, primary human skin fibroblasts (with high proliferation rate) were seeded onto decellularised human kidney scaffolds (Figure 5.10). Different numbers of skin fibroblasts (1 x 10^5, 2 x 10^5 and 4 x 10^5 cells/scaffold) were seeded onto the scaffolds and incubated for 2 weeks. More primary skin fibroblasts were available than primary renal fibroblasts, however, the lowest number of skin fibroblasts

![Figure 5.10 Effect of human skin fibroblast number on repopulation of human kidney cortex ECM scaffolds.](image)

Primary human skin fibroblasts were seeded on the surface of decellularised human kidney scaffolds and incubated for 2 weeks. 1 x 10^5 cells/scaffold (A), 2 x 10^5 cells/scaffold (B) and 4 x 10^5 cells/scaffold (C) were seeded. Scaffolds were fixed, sectioned and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Red square highlights the cells in the scaffold and the high-power image of these cells are shown in the insert (bottom right corner). Magnification 5x. N=1 experiment with 3 scaffolds/condition. Representative images of 10 images/scaffold are shown.
tested was less than the highest number of renal fibroblasts. In all scaffolds, fibroblasts were observed to repopulate decellularised scaffold. Even with the lowest number of skin fibroblasts were seen attached on the edge of the matrix (Figure 5.10A) and in scaffolds seeded with higher numbers of cells, skin fibroblasts appeared within the scaffold (Figure 5.10C). This suggested that the lack of attachment and growth of primary renal fibroblasts when seeded on the scaffold was not a generic feature of primary fibroblasts.

Given the poor repopulation of scaffolds with primary human renal interstitial fibroblasts, a conditionally-immortalised human kidney fibroblast line (tsHKF) was tested (252). Different numbers of tsHKF ($1 \times 10^5$, $2 \times 10^5$ and $4 \times 10^5$) were seeded onto scaffolds and incubated at 33°C (the permissive temperature at which cells continue to proliferate) for 2 weeks. The extent of repopulation of the scaffold was dependent on starting cell number and the highest number of tsHKF seeded produced the most extensive repopulation (Figure 5.11C). Interestingly, tsHKFs appeared to aggregate in limited areas of the scaffold, primarily where the scaffolds were slightly folded (created by the way that scaffold was inserted into round-bottom wells) (Figure 5.11). There were also some individual fibroblasts scattered in the matrix and attached to the surface of the matrix. For all subsequent experiments, $4 \times 10^5$ tsHKF cells were seeded on the surface of scaffold for 2 weeks.

Figure 5.11 Effect of tsHKF number on repopulation of human kidney cortex ECM scaffolds.

tsHKF were seeded onto the surface of the scaffolds at $1 \times 10^5$ cells/scaffold (A); $2 \times 10^5$ cells/scaffold (B) or $4 \times 10^5$ cells/scaffold (C) and incubated at 33°C for 2 weeks. Scaffolds were fixed, sectioned and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=1 experiment with 3 scaffolds/condition. Representative images of 10 images/scaffold are shown.
The effect of tsHKF on the matrix was also examined by H&E and PSR staining. tsHKF had a significant impact on matrix structure around the areas of cell aggregation (Figure 5.12D-F, G-I). Fibroblasts seemed to contract or remodel the matrix producing new matrix (collagens) (Figure 5.12G, H, I). At this level of analysis, it is not possible to distinguish between these two processes, but there is a marked increase in density of the ECM around the cell aggregates.

Figure 5.12 Comparison of the histology of intact kidney, decellularised ECM scaffold and scaffolds repopulated with tsHKF.
Normal human kidney (A-C). Decellularised scaffold (D-F). Decellularised scaffold repopulated with tsHKF (4 x 10^5 cells/scaffold) and incubated at 33°C for 2 weeks (G-I). Samples were fixed, sectioned and stained with H&E and PSR. H&E staining (A, D, G). Brightfield image of PSR staining (B, E, H). Polarised light image of PSR staining (C, F, I). Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=3 experiments using scaffolds from 3 different kidneys (3 scaffolds/condition within each experiment). Representative images of 10 images/scaffold are shown.
To further analyse the effect of matrix on tsHKF cells, cell proliferation (Ki67), apoptosis (cleaved Caspase-3), and expression of mesenchymal markers (Vimentin and α-SMA; α-SMA is also a marker of myofibroblast differentiation) were compared in cells seeded on glass vs decellularised scaffolds. Seeding cells on the scaffolds significantly reduced proliferation 95.16±0.45% Ki-67-positive cells on glass compared with 56.32±1.93% on the scaffold (Figure 5.13A-D, I). Analysis of cleaved caspase-3 by conventional 2D IFL was confounded by high background, possibly due to the high density of the ECM round the cell aggregates. Therefore, confocal imaging, with a better spatial resolution, was used to analyse cleaved caspase-3 expression in tsHKF (548 tsHKF cells were counted from 10 images) seeded on the decellularised matrix in order to provide some rationale for how apoptosis changed in tsHKF on the matrix. Expression of cleaved caspase-3 seemed to be increased from 11.19±0.58% positive cells on glass to 36.74±4.71% on the scaffold (Figure 5.13F-J). However, due to the fact the different techniques (conventional and confocal IFL) were used to analyse cleaved caspase-3 expression of tsHKFs seeded on glass and scaffold, statistical analysis could not be used to determine the significance. Mesenchymal markers showed different patterns of expression. All tsHKF showed high cytoskeleton expression of vimentin on both glass (99.89±0.06% positive cells) and scaffold (99.79±0.11%) with no difference (Figure 5.14A-D, I). The similar pattern of vimentin expression on renal fibroblasts has been described previously (449, 450). Cells can express α-SMA in a diffuse cytoplasmic pattern or incorporated into stress fibres (characteristic of contractile myofibroblasts) (91). When both diffuse and stress-fibre incorporated α-SMA were considered, almost all tsHKF expressed α-SMA both glass (96.57±0.29% positive cells) and matrix (97.84±0.36%) (Figure 5.14E-H, L). However, when only stress fibre-incorporated α-SMA was evaluated, expression of in tsHKF seeded on glass was very low (2.12±0.28% positive cells) (Figure 5.14E-H, K) while 30.99±2.06% fibroblasts were positive on the decellularised matrix (Figure 5.14E-H, K). This is consistent with the qualitative
Figure 5.13 Effect of glass substrate vs decellularised scaffold on tsHKF proliferation and apoptosis.

tsHKF were seeded onto either glass coverslips or decellularised scaffold (4 x 10^5 cells/scaffold) and incubated at 33°C for 2 weeks. Samples were then fixed and processed for IFL. Sections were incubated with Ki67 (B, D) and cleaved caspase-3 (G, I) antibody or an IgG control for antibody specificity (A, C, F, H) at an equivalent concentration to the primary antibody, nuclei were stained with DAPI. tsHKF on glass (A, B, F, G); tsHKF on scaffold (C, D, H, I). Images of conventional IFL (A-D, F, G) and confocal microscopy (H, I) microscope. Percent Ki67 (E) positive cells (>20 images/condition) were quantified. Representative images of 20 images/condition are shown. Percent cleaved caspase-3 (J) positive cells (3 to 4 images/experiment) were quantified. Arrows indicate positive cells. Representative images (3 to 4 images/experiment) are shown. N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds(condition within each experiment). Magnification: 20x (A-D, F, G); 40x (H, J). ****p<0.0001. Error bars: Mean + SD.
Figure 5.14 Effect of glass substrate vs decellularised scaffold on the expression of mesenchymal markers in tsHKF.

(tsHKF) were seeded onto either glass coverslips or decellularised scaffold (4 \times 10^5 cells/scaffold) and incubated at 33°C for 2 weeks. Samples were fixed and processed for IFL. Vimentin expression on glass (A, B) vs decellularised scaffold (C, D). α-SMA expression on glass (E, F) vs decellularised scaffold (G, H). Sections/coverslips were incubated with vimentin (B, D) and α-SMA (F, H) antibody, and IgG control (A, C, E, G) for antibody specificity (IgG was used at an equivalent concentration to the primary antibody). nuclei were stained with DAPI. Percent vimentin-positive (I) and α-SMA positive cells showing diffuse cytoplasmic expression (J) and stress fibre expression (K) on scaffold vs glass (>10 images/condition were quantified). Arrows indicate positive cells. N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds/condition within each experiment); representative images of 20 images/condition are shown). A-H: Magnification: 20x. ns: not significant, *: p<0.05, **: p<0.01, ****: p<0.0001. Error bars: Mean + SD.

observations of sections of tsHKF in the scaffolds, which suggested cells were contracting the scaffold.

In summary, the results indicate that tsHKF tended to form several aggregates within the matrix with a smooth surface across the aggregate or individual tsHKF stay isolated within the matrix or along the surface of the scaffold. It should also be noted that all cells were seeded into scaffolds in round-bottomed plates in which the scaffold was partially folded in a horseshoe shape. tsHKF retained this structure even when the matrix was transferred to a larger well. This is different from the scaffold seeded with epithelial cells where the scaffold flattened after transfer into a 24-well plate. Marker studies showed that seeding on the matrix suppressed fibroblast proliferation even at the permissive temperature with a slight increase in apoptosis and in the number of cells expressing α-SMA-positive stress fibres.

5.3.3 Human podocytes on decellularised human kidney cortex ECM

Within the renal cortex, another distinct ECM structure is within the glomerulus. The effect of ECM scaffolds on podocytes was tested using the well-described conditionally-immortalised podocyte cell line, ciPod (253). Initial repopulation experiments used podocytes differentiated for 2 weeks at 37°C according to the established protocol for ciPod differentiation (253). Scaffolds were seeded with a range of cell numbers (5 \times 10^4, 1 \times 10^5 and 2 \times 10^5 cells/scaffold) and incubated for 2 weeks
However, even with $2 \times 10^5$ cells/scaffold, there was no evidence of repopulation (Figure 5.15C). An alternative approach was tried where undifferentiated ciPod were grown at 33°C to 80% confluence then differentiated at 37°C for 2 weeks then seeded on the surface of scaffold and incubated for 2 weeks at 37°C. $5 \times 10^4$ cells/scaffold (A), $1 \times 10^5$ cells/scaffold (B), $2 \times 10^5$ cells/scaffold (C). Scaffolds were fixed, sectioned and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=1 experiment with 3 scaffolds/condition. Representative images of 10 images/scaffold are shown.

podocytes grown at 33°C (the permissive temperature) were seeded onto the matrix, incubated overnight to promote cell attachment and then incubated for 2 weeks at 37°C. However, again there was no evidence of cells repopulating the scaffold regardless of the number of podocytes seeded (Figure 5.16A-C). In the third protocol, podocytes grown at 33°C were seeded on the decellularised matrix and incubated for 2 weeks at 33°C (Figure 5.17). The highest number of podocytes ($4 \times 10^5$ cells/scaffold) seeded
Figure 5.17 Effect of undifferentiated ciPod cell number on repopulation of decellularised human kidney cortex scaffolds.

CiPod were grown at 33°C to 80% confluence then seeded on the surface of scaffolds and incubated at 33°C for 2 weeks. 1 x 10^5 cells/scaffold (A), 2 x 10^5 cells/scaffold (B), 4 x 10^5 cells/scaffold (C). Scaffolds were fixed, sectioned and stained with H&E. Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=1 experiment with 3 scaffolds/condition. Representative images of 10 images/scaffold are shown.

showed the most extensive repopulation (Figure 5.17C). Therefore, for all subsequent experiments, 4 x 10^5 ciPod cells were seeded on the surface of scaffold and incubated at 33°C for 2 weeks.

Podocytes appeared to line the surface (basement membrane) of larger lumens and form clusters attached to the basement membrane of small lumens (Figure 5.17B, C), however, there was no evidence of podocytes within glomeruli. The behaviour of the podocytes on the scaffolds resembled that of the epithelial cells (line the basement membrane of large lumens), and there was no evidence of remodelling the matrix (PSR staining) as seen with fibroblasts (Figure 5.18).

Marker studies were also conducted on ciPod seeded on the ECM scaffolds. Expression of Ki67 was reduced in ciPod seeded on scaffolds (64.66±1.57% positive cells) compared with glass (98.79±0.16% positive cells) (Figure 5.19A-D, I) while cleaved caspase 3 expression was slightly increased in cells seeded on the matrix (11.17±0.77% vs 3.31±0.23% positive cells) (Figure 5.19E-H, J). Staining with podocyte markers, synaptopodin, nephrin, Wilms’s tumour-1 (WT1), showed no change in nephrin expression (in a weak diffuse pattern) on the two substrates, however, there is no staining in the cell nuclei as expected for differentiated podocytes (253) (Figure 5.20E-H, J). Synaptopodin has been reported to be present in podocytes with diffuse
cytoplasmic expression (undifferentiated) or incorporated into the cytoskeleton (differentiated) (253). ciPod at 33°C on glass showed diffuse cytoplasmic expression but no cytoskeletal localisation (Figure 5.20A-D, I) while on the decellularised matrix 61.01±3.27% of cells showed cytoskeletal expression but no diffuse cytoplasmic expression (Figure 5.20A-D, I). The expression of WT1 was significantly increased in podocytes seeded on the matrix (11.84±1.12% positive cells) compared with glass (1.27±0.14%) (Figure 5.21 A-D, I). On both glass and the scaffolds, almost all cells

![Image of histology comparison](image)

**Figure 5.18** Comparison of the histology of intact kidney, decellularised ECM scaffolds and scaffolds repopulated with ciPod cells. Normal human kidney (A-C). Decellularised scaffold (D-F). Decellularised scaffold repopulated with ciPod cells (4 x 10^5 cells/scaffold) and incubated at 33°C for 2 weeks (G-I) were fixed, sectioned and stained with H&E and PSR. H&E staining (A, D, G). Bright-field image of PSR staining (B, E, H). Polarised light image of PSR staining (C, F, I). Seeded cells in the scaffold were indicated by arrow. Magnification: 5x. N=3 experiments using scaffolds from 3 different kidneys (3 scaffolds/condition within each experiment). Representative images of 10 images/scaffold are shown.
Figure 5.19 Effect of glass substrate vs decellularised scaffold on undifferentiated ciPod cell proliferation and apoptosis.

ciPod cells were seeded on glass coverslips or decellularised ECM scaffolds (4 x 10^5 cells/scaffold, incubated for 2 weeks at 33°C) and then fixed for IFL. Ki67 expression on glass (A, B) vs decellularised scaffold (C, D). Cleaved caspase-3 expression on glass (E, F) vs decellularised scaffold (G, H). Sections/cover slips were incubated with Ki67 antibody (B, D), cleaved caspase-3 (F, H) or IgG control (A, C, E, G) for antibody specificity (IgG was used at an equivalent concentration to the primary antibody), nuclei were stained with DAPI. Percent Ki67-positive (I) and cleaved caspase-3-positive (J) cells on scaffold vs glass (>=20 images/condition were quantified). Arrows indicate positive cells. The insert (bottom left corner) shows the high-power image of weak positive cells. N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds/condition within each experiment), representative images of 20 images/condition are shown. A-H: Magnification: 20x. ****: p<0.0001. Error bars: Mean + SD.

seeded showed a basal level of diffused cytoplasmic α-SMA expression (Figure 5.21 E-H, J).

In summary, differentiated ciPod failed to repopulate that ECM scaffolds but undifferentiated cells maintained at the permissive temperature successfully repopulated the scaffolds. The behaviour of podocytes was similar to that of proximal tubular epithelial cells. Cells appeared to align along the basement membranes of tubular lumens. When cells aggregated in the small lumens, qualitative assessment suggested they formed clusters with some cells attached to the basement membrane while others were attached to another cell. At the permissive temperature, the proliferation of ciPod cells was reduced by the matrix with increased apoptosis. The matrix could also enhance the expression of some markers of the podocyte, but not all of them suggesting that ECM did not support complete differentiation of the cells.
Figure 5.20 Effect of glass substrate vs decellularised scaffold on the expression of podocyte-specific markers in ciPod.

ciPod cells were seeded on glass coverslips or decellularised ECM scaffold (4 x 10^5 cells/scaffold and incubated for 2 weeks at 33°C), samples were then fixed and processed for IFL. Synaptopodin expression on glass (A, B) vs decellularised scaffold (C, D). Nephrin expression on glass (E, F) vs decellularised scaffold (G, H). Sections/cover slips were incubated with synaptopodin (B, D), nephrin (F, H) antibody or IgG control (A, C, E, G) for antibody specificity (IgG was used at an equivalent concentration to the primary antibody), nuclei were stained with DAPI. Percent synaptopodin-positive cells showing cytoskeletal expression (J), nephrin-positive (I) cells on scaffold vs glass (>20 images/condition were quantified). Arrows indicate positive cells. The insert (bottom left corner) shows the high-power image of weak positive cells. N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds/condition within each experiment); representative images of 20 images/condition are shown. A-P: Magnification: 20x. ns: not significant, *** p<0.001, **** p<0.0001. Error bars: Mean ± SD.
Figure 5.21 Effect of glass substrate vs decellularised scaffold on the expression of podocyte-specific markers in ciPod.

ciPod cells were seeded on glass coverslips or decellularised ECM scaffold (4 x 10^5 cells/scaffold and incubated for 2 weeks at 33°C), samples were then fixed and processed for IFL. WT1 expression on glass (A, B) vs decellularised scaffold (C, D). α-SMA expression on glass (E, F) vs decellularised scaffold (G, H). Sections/coverslips were incubated with WT1 (B, D) and α-SMA (F, H) antibody or IgG control (A, C, E, G) for antibody specificity (IgG was used at an equivalent concentration to the primary antibody), nuclei were stained with DAPI. Percent WT1-positive (I) and α-SMA-positive (J) cells on scaffold vs glass (>20 images/condition were quantified). Arrows indicate positive cells. The insert (bottom left corner) shows the high-power image of weak positive cells. N=3 experiments using scaffolds from 3 different kidneys (3 coverslips or 3 scaffolds/condition within each experiment); representative images of 20 images/condition are shown. A-P: Magnification: 20x. *** p<0.001, **** p<0.0001. Error bars: Mean + SD.

5.4 Discussion

As described in the Introduction (and in Chapter 1, Section 1.4.4) a variety of cell types have been used to repopulate kidney ECM scaffolds from a variety of species. The renal epithelial cells were used to repopulate the decellularised rat and pig kidneys by both perfusion of whole kidney scaffold and static culture of decellularised tissue cubes (196, 219, 230). However, few studies have looked at the behaviour of human cells on human kidney scaffolds, such as human iPSC derived endothelial cells (162), human primary kidney adult stem cells (235), human kidney peritubular microvascular endothelial cells and human umbilical vein endothelial cells (168). The aim of our study was to examine the effect of decellularised human kidney ECM scaffolds on different human renal cell types to determine whether there was cell-type specific localisation within the scaffold and/or differential effects of the scaffold on the different cell types to better understand how the renal ECM regulates renal cell function. This study used three cell types from the glomeruli and tubulointerstitium. The predominant epithelial cell type in the renal cortex is the proximal tubular epithelium, therefore, the human proximal tubular epithelial cell line (HK2) was selected as a representative line. Podocytes are a key cell type in the glomerulus and in control of blood filtration. Thus, conditionally immortalised human podocytes were selected. Finally, interstitial fibroblasts were selected as they are a key cell type in maintaining normal kidney structure and play an
important role in fibrosis in multiple kidney diseases. None of the previously published studies has directly compared the behaviour of different renal cell types on human kidney ECM scaffolds. In addition, our study examined the effect of scaffolds generated from the same kidney on different cell types thereby any potential variation induced by the scaffold itself could be kept minimal. The effect of ECM vs glass on proliferation, apoptosis and differentiation of the different cell types was also compared. It should be noticed that in this study the glass was not coated (e.g. with collagen 1), so the comparison is between 2D cultures on glass only and culture in a 3D scaffold. It would be of interest to compare a 2D human kidney ECM matrix with the 3D scaffold.

In the present study, the HK2 proximal tubule epithelial cell line repopulated the scaffolds lining the tubular lumen-like structure of decellularised matrix with a monolayer of cells resembling the single layer of epithelial cells lining the nephron of the intact kidney. There were no differences observed in HK2 cells seeded on scaffolds derived from 3 different human kidneys. It is possible that there is preferential repopulation of tubules that are cut through at the scaffold surface, creating an opening extending into the body of the cubes and into which cells can readily migrate. It could be speculated that cell proliferation, migration or both contribute to the repopulation of tubule-like structure, however, it is impossible to differentiate between them in our current experimental settings in any cell types. Previously published studies showed that proximal tubule cells isolated from rat neonatal kidneys or adult pig kidneys could form tubule-like structures in the decellularised scaffold (219, 230). Other investigators have also reported a similar pattern of recellularisation of rat kidney ECM scaffolds with a mixture of human cortical tubular epithelial cells (a mixture of proximal and distal epithelial cells) (196, 219, 230). These results suggest the epithelial cells can attach to the matrix and arranged themselves along the basement membranes. When accessing repopulation of HK2 cells using H&E staining, none of the studies of epithelial cells showed any marked changes in the matrix (196, 219, 230). Poornejad and colleagues used PSR to stain the decellularised kidney matrix which showed a similar distribution of
collagens to that seen with decellularised scaffold in our study, prior to repopulation, however, the PSR staining of matrix seeded with epithelial cells was not reported, therefore there is no comparator for our study (220). In our study, the matrix appeared to suppress the capacity of an immortalised cell line to proliferate and increased cell apoptosis with increased differentiation markers at 14 days. This might suggest that our matrix could push the differentiation of HK2 cells while suppressing their proliferation capability as normal proximal tubular epithelial cells do not proliferation in kidney, and increased apoptosis might associate with the death of cells which could not differentiate. Similar results were observed when Abolbashari and colleagues perfused primary pig kidney epithelial cells (a mixture of proximal, distal tubular and collecting duct cells) into decellularised whole pig kidney matrix (230). Abolbashari and colleagues examined cell proliferation and apoptosis using PCNA staining and TUNEL assay, respectively (230), but they did not compare this result with cells seeded on other substrates such as glass or plastic. Similar to our study, primary pig proximal tubule epithelial cells were PCNA-positive at day 14 after seeding suggesting that the matrix supports proliferation of the cells over the 2 week period (230), although no time-course study or quantitative data were reported. Similar to the trend of increased cell apoptosis in our study, Abolbashari and colleagues also demonstrated a significant increase in cell death between 14 (~6% positive cells) and 28 days (~12%) using TUNEL assay (230). However, in our study, different level of apoptosis of HK2 cells was observed (~9.45%) at 2 weeks incubation which might reflect the difference in culture system between our study (static culture) and that of Abolbashari et al (prefusion), and the difference between primary cells with immortalised cell lines. Markers of epithelial cell were also used to analyse repopulation of kidney matrix in many studies (219, 230, 451). In our study, staining of HK2 cells on decellularised human kidney matrix for markers of proximal tubular epithelial cells (LTL and AQP1) suggested increased expression of these differentiation markers. A similar expression of LTL has been reported in primary proximal tubule epithelial cells seeded on hydrogel (451) suggesting that the 3D matrix and hydrogel may provide clues to
promote expression of this marker. In our scaffolds, more LTL expression was observed in cells close to the surface of the scaffold and the repopulation of the scaffold is largely confined to the area at immediately below the surface (representing potentially around 20% of total cube volume). It could be speculated that in static culture nutrient and oxygen supply for seeded cells is better near the surface of the scaffold which may support better differentiation. In our study, increased expression of AQP1 was observed in the proximal tubular epithelial cells seeded on the decellularised human kidney cortex cubes. Similar to our results, HK2 cells seeded onto decellularised rat kidney slices showed increased AQP1 expression compared with tissue culture plastic (452). In Song et al study, rat neonatal renal epithelial cells (a mixture of proximal and distal tubular epithelial cells) perfused into decellularised whole rat kidney matrix also displayed a differentiated phenotype with expression of AQP1 (219). In another study, Abolbashari et al maintained high percentage of AQP1-positive cells in primary pig kidney epithelial cells (a mixture of proximal, distal tubular and collecting duct cells) perfused into decellularised whole pig kidney matrix compared to culture on tissue culture plastic (72% AQP1-positive cells on plastic and 75% on matrix) (230). Taken together, these data show that decellularised kidney matrix may promote or maintain the proximal tubular epithelial cell differentiation. After using epithelial cell-specific markers, mesenchymal markers, vimentin and α-SMA, were also used to examine HK2 cell after seeded onto matrix. Colocalisation study of our matrix seeded with HK2 cells showed that epithelial cells could express both proximal tubular epithelial marker (LTL) and mesenchymal marker (α-SMA) at same time. It should be noted that vimentin expression was already reported when the cell line was established (453) and the similar pattern of vimentin expression was also reported (449). Perhaps surprisingly although seeding on ECM scaffolds appeared to promote HK2 cell differentiation with increased the expression of proximal tubular epithelial cell markers, compared with plating on glass, cells continued to express mesenchymal markers, vimentin and α-SMA, suggesting the differentiation of HK2 cell remained incomplete which may relate to the continued proliferation. Similar
low level and diffused α-SMA expression in the cytoplasm of HK2 cells has also been reported in previous studies (454-456). It could be speculated that the full differentiation of this cells (high expression of specific markers in all cells) may require additional factors, for examples, complete stop of proliferation (reversal of viral transformation), or other cell types to present in the environment. In summary, our results provide the first study of human proximal tubular epithelial cells plated on decellularised human kidney cortex matrix and suggest that the matrix scaffold supports HK2 cell growth and differentiation with the formation of tubule-like structures which may be important in future development of multicellular tissue mimetic technique as a platform to understanding the interaction between cell and matrix.

Primary normal human kidney interstitial fibroblasts failed to repopulate the decellularised kidney scaffold. Although renal interstitial fibroblasts from two human kidneys were tested, it did not seem seeding primary human kidney interstitial fibroblast is a viable option. Due to the difficulty of culturing primary human kidney fibroblast, another human primary fibroblast (skin fibroblast) was seeded onto the scaffold. Results indicated that the behaviours of human kidney interstitial fibroblasts did not appear to be a general characteristic of primary cultures of fibroblasts since primary human skin fibroblasts attached and repopulated the matrix. However, this difference is not explained by the number of cells seeded. Compared to the renal fibroblasts, the skin fibroblasts have a high proliferative capacity thus failure to repopulate the scaffold may reflect the limited proliferative capacity of primary human kidney fibroblasts. However, it is still unclear why the human kidney fibroblasts do not appear to attach to the matrix.

A number of other studies looking at fibroblast repopulation of kidney matrices have used immortalised cell lines (457, 458). Willenberg and colleagues perfused immortalised mouse lung fibroblasts into decellularised pig kidney via the renal artery and showed that after 3 days cells were present in the peritubular capillary networks and were able to grow on the pig kidney matrix (457). In another study, a rat kidney fibroblast cells line (NRK-49F) was seeded onto decellularised mouse kidneys slices (458). Cell
tracker and PCNA staining indicated that immortalised rat kidney fibroblasts could repopulate and proliferate on mouse kidney matrix. Based on these two sets of results and our observation in skin fibroblasts, this may suggest that rapid proliferation at the time of seeding may be the key for successful repopulation. To determine whether the low proliferation rate might be an issue in repopulating human kidney ECM scaffolds with human kidney fibroblasts, conditionally immortalised human kidney interstitial fibroblasts (tsHKF) (252) were tested using both permissive and non-permissive temperature. After 2 weeks, there are no histological differences observed between tsHKF cultured under permissive and non-permissive temperature. Therefore, the requirement for cell proliferation is reinforced by the fact that tsHKF were not pre-differentiated before seeding, therefore, they had a high proliferation rate and repopulated the scaffold successfully. When the similar number of tsHKF (compared with seeded normal human kidney fibroblast) were initially seeded onto the matrix, tsHKF adhered and repopulated to the matrix suggesting that the number of cells seeded onto the matrix is not the issue that primary fibroblasts failed to repopulate the matrix. Again, this reinforces that the key issue is probably the proliferation. These tsHKF cells attached and grew on the decellularised scaffolds derived from 3 different kidneys. The behaviour of tsHKF was significantly different from that of either HK2 or ciPod cells. The fibroblasts appeared to form large aggregates of cells in a few regions of the scaffold which scaffolds were slightly folded in the round-bottom plate, and there also appear to be individual fibroblast on the surface and scattered through the ECM. During cell seeding, single cells suspension of fibroblast was confirmed using light microscopy (data not shown). However, this observation did not rule out the possibility that a few cells proliferated to make an aggregate or fibroblasts migrate to each other to form an aggregate after seeded onto the matrix. PSR staining of total collagens revealed dense staining around both the fibroblast aggregates and individuals, suggesting that tsHKF were either making more collagen or contracting the matrix to compact it. This type of matrix remodelling/contraction has neither been observed in seeded human skin fibroblast in
our study nor been reported in fibroblasts (immortalised cell lines) from other organs seeded onto/into kidney matrix in a previous study (457). Although Fu and colleagues perfused rat kidney fibroblasts (NRK49F) into decellularised mouse kidney, no histology was reported so it was not possible to assess remodelling of the matrix, however, the even distribution of cells through the matrix in the published IFL images suggests no clustering/matrix contraction (458). When tsHKF on the scaffolds were stained for vimentin and α-SMA, the level of vimentin expression was unchanged and the number of cells with α-SMA positive stress-fibres (31%) was significantly increased suggesting that these tsHKF were differentiated into contractile myofibroblasts. However, the reason for tsHKF to behave differently compared with epithelial cells and podocytes are still unclear. It is possible to speculate that this could be because these cells have inherent contractility, and they do remodel the matrix when there is a matrix which can be easily manipulated. It could also be speculated that when interstitial fibroblasts are plated into the matrix at relatively high density, a pseudo wound healing response is initiated. Moreover, the effect of the configuration of the scaffold may contribute to this situation because the partial folding of the matrix in round bottom plate during cell seeding could create a ‘fold’ in which tsHKF tend to adhere and contract the matrix around them which was not observed in epithelial cells but these cells lack inherent contractility. Although it is still unclear why tsHKF formed aggregates, it could be speculated that this might relate some unknown characteristic of this conditionally immortalised cell type. In summary, our results provide the first report of plating different human fibroblasts (primary normal kidney interstitial fibroblasts, primary skin fibroblasts and tsHKF) on human decellularised kidney cortex matrix and suggest that decellularised human kidney matrix supports the attachment and growth of primary human skin fibroblasts and tsHKF but not of primary human renal fibroblasts. On the scaffolds, tsHKFs form cell clusters, myofibroblast differentiation is enhanced and there is remodelling/contraction of matrix which may suggest the matrix drive myofibroblast differentiation and matrix remodelling although it is unclear why they formed cell aggregates. These results demonstrate that
decellularisation technique is a good platform to study cell-matrix interaction, and highlight the need for understanding the mechanism of cells interact with matrix and why same cell type (fibroblast) from different organ (skin and kidney) behave differently on same matrix.

Conditionally-immortalised podocytes (ciPod) were seeded on the decellularised scaffolds using the same method as for epithelial cells and fibroblasts. Initial studies used differentiated podocytes (incubated for 14 days at 37°C) however these cells failed to repopulate the matrix. Based on the findings with fibroblasts with low proliferative capacity, this may be due to the limited proliferation of differentiated ciPod (253). In addition, this is not the issue of the number of cells seeded, as the same number of undifferentiated ciPod could repopulate the matrix when cultured at the permissive temperature. It is similar to epithelial cells and fibroblasts, both proliferation and migration may contribute to the repopulation of the matrix, however, could not be differentiated in this study. In contrast with differentiated podocytes, undifferentiated podocytes (grown at 33°C) were plated onto the scaffolds and incubated at the permissive temperature (33°C), the podocytes appeared to line the large lumens within the scaffold forming a monolayer of cells. In smaller tubular profiles, podocytes also formed clusters in our study which is similar to data obtained when conditionally-immortalised mouse podocytes were perfused into decellularised whole rat kidney scaffold via the ureter (228). In this study, the majority of mouse podocytes formed clusters and attached to tubular basement membranes (228). The lining of large tubular profiles resembled the pattern of repopulation observed with HK2 on the human kidney ECM scaffolds, and it could be suggested that this observation reflects the epithelial nature of podocytes which are derived from epithelial precursors during renal development (32). Staining CiPod on the scaffolds with podocyte markers revealed some interesting results. When this conditionally-immortalised podocyte cell line was established, a series of markers were used, including WT1, synaptopodin, nephrin (253). The same markers were used in the present study and showed that the scaffold promoted podocyte differentiation with cell
nuclear expression of WT1, appearance of synaptopodin along actin filaments. WT1 was not expressed in podocytes on glass but nuclear expression increased to 12% on the matrix. As described by Saleem et al., there was weak expression of synaptopodin in undifferentiated podocytes on glass (253). In our study, on the decellularised scaffold, more than 55% of the cells expressed synaptopodin which appeared to align along actin filaments, a pattern which was only observed in differentiated podocytes according to the original paper (253). However, the expression of nephrin in podocytes on the matrix was weak and diffuse which is consistent with undifferentiated podocytes (253). These results suggest that the decellularised kidney matrix can promote differentiation of undifferentiated podocytes at 33°C although this was not complete. The incomplete differentiation might reflect the continuing high level of proliferation (64.66±1.57% of podocytes seeded on the matrix are proliferative) (Figure 5.19 A-D, I). If the proliferation could be slowed down by increased temperature and removal of supplements containing insulin, transferrin and selenium from medium, it is possible that the cell differentiation will be further enhanced. Like epithelial cells, it may also be possible that the full differentiation of ciPod requires additional factors including the presence of adjacent cell types. This is showed by Byron and colleagues that co-culture of glomerular endothelial cells and podocytes could generate matrix similar to normal glomerular matrix with better cell differentiation (34). Weak, α-SMA expression was detected in all podocytes on both glass and the scaffold, which is consistent with a previous study (459). Although podocytes have not previously been seeded on decellularised human kidney matrix, some studies have grown podocytes on artificial basement membrane and hydrogels (460-462). Slater et al. showed that the same human undifferentiated podocytes (ciPod) could grow on electrospun collagen (collagen I) nanofiber membranes for 1 week where they formed monolayers (460) which is consistent with our observations. Conditionally-immortalised mouse podocytes seeded on thin sheets of hydrogel (1-5µm) in static culture showed 12.6% cell death which was similar to our result of 11.17% cell death. It is unclear why this level of cell apoptosis happened, however, this might be due to the
matrix, hydrogel or artificial basement membrane driving cell differentiation and cells that cannot differentiate undergo apoptosis (461). However, no cell marker studies were reported in the published studies, so a direct comparison is not possible. Li et al. reported increased expression of nephrin and synaptopodin in conditionally-immortalised mouse podocytes on collagen IV-coated, microporous polyethylene terephthalate membranes compared with collagen I-coated substrate (462). Our data is consistent with this finding in that podocytes lined the basement membrane-like structures forming a monolayer. A recent study showed that there was increased podocin expression in conditionally-immortalised human podocytes (the same cell line as used in our study) when 10% pig bladder matrix which contains collagen IV and other matrix proteins) was added into a hydrogel (463) compared to hydrogel alone. These results suggest that a variety of ECMs and hydrogel could support the growth of podocytes to form a monolayer/epithelium-like structure, and collagen IV contained in the basement membranes could be one of the drivers for podocyte differentiation. Our study provides the first report of human podocytes seeded on human decellularised kidney cortex matrix and suggest that decellularised human kidney matrix can drive differentiation of podocytes with reduced proliferation and increased apoptosis, even at the permissive temperature indicating a potent effect of matrix. These results suggest that the ECM plays an important role in regulating cell behaviour and decellularised matrix could be used as a platform to develop a better the cell culture system.

In this chapter, a method was established to recellularise human kidney ECM scaffolds, and three different human kidney cell types were used to repopulate the scaffolds. Our data suggest human kidney cortex matrix support/promotes differentiation of both human proximal tubular epithelial cells and human podocytes. Interestingly, when tsHKFs were seeded onto decellularised matrix, contraction of the matrix was observed, and a significant proportion of tsHKFs turned into contracting myofibroblasts. In conclusion, these data showed human kidney matrix could support the growth and drive differentiation of these three different cell types separately, and it will be interesting in
the future to look at more complex cultures to form the basis for developing complex multicellular tissue mimetics as a platform to understanding the role of the ECM in regulating cell behaviour in kidney and to use for drug screening.
Chapter 6

General discussion and future work
6.1 General discussion

The aim of this thesis was to characterise the ECM of the human kidney cortex and investigate how this matrix may regulate renal cell behaviour. This study focussed on the cortex as it represents a key area affected in CKD. An understanding of the normal ECM and its regulatory functions is important for understanding the pathological changes that occur in disease. The first step was to define the composition of the ECM in the human kidney cortex. The kidney cortex can be divided into two main compartments, the glomeruli and the tubulointerstitium. The composition of the glomerular (G) ECM has been studied previously using ECM enrichment and MS-based proteomic techniques (139, 167) providing a global profile of this matrix. However, to date, all of the information on the composition of tubulointerstitial (TI) ECM has come from candidate-based approaches (55-58). Therefore, an approach similar to the one used to profile the G matrix (139) was adopted to profile the TI matrix. The data in Chapter 3 provides the first detailed characterisation of the human TI matrix as well as the first direct comparison of human G matrix with TI matrix from the same kidneys. Using the HMDB, 140 proteins were identified in the TI ECM and 164 proteins in the G matrix. Proteins identified in the TI ECM were searched for in published literature on Pubmed to determine whether they had previously been identified in the kidney and/or the tubulointerstitium of any species. This extensive literature review revealed 2 proteins (CASP14 and SERPIND1) which had not previously been reported in the kidney of any species. This search also revealed 4 proteins (CCT2, DSG1, MATN2 and PLOD1) that had not previously been identified in the human kidney despite being reported to be present in the kidney of other species including mouse and rat (310, 314, 321, 464). Among the other proteins identified, 75 were localised to the TI matrix of any species for the first time. When comparing the components of the TI matrix with the G matrix, 126 proteins identified were common to both compartments, covering a broad range of HMDB categories. There were 14 and 38 ECM proteins were identified only in the TI and G matrix, respectively (Chapter 3, Section 3.3.5 and 3.4) indicating that a large proportion of the proteins in the TI and G matrix are
shared despite the differences in their structure and function. This suggests that the fundamental framework of TI and G matrices are similar and that differences between G and TI matrices may come from differences in the amount and localisation of proteins and/or the unique proteins in each compartment. Comparing our data on the human G matrix with previously published proteomic data, most of the core matrisome proteins (44 out of 48 basement membrane and other structural proteins) identified by Lennon et al. (139), and all core matrisome proteins identified by Hobeika et al. (167) were also present in our study. The main differences between our study and the studies by Lennon et al. and Hobeika et al. were related to the ECM-associated proteins. As discussed previously (Chapter 3 Section 3.4.2.1), there were newly identified proteins which covered a range of HMDB protein categories, with the majority being ECM-associated proteins (38 proteins). Moreover, an additional 2 basement membrane proteins (LTBP4 and PXDN) and 13 other structural proteins (COL5A2, COL7A1, COL14A1, COL16A1, EFEMP1, FBLN5, IGFBP7, MATN2, MFAP4, MFGE8, OGN, PRG2 and VCAN) were identified which were not reported by either Lennon et al. or Hobeika et al. (139, 167). Interestingly, most of the core matrix proteins (basement membrane proteins, other structural proteins), which were identified in the G ECM in our study, and in the two previous studies (139, 167), were also observed in mouse lung matrix apart from LAMB1 (465) suggesting that core matrix proteins may be similar between different organs and different species. The data from our study enhance our understanding of the ECM of the normal human kidney TI and illustrate the complex composition of this matrix. These data also expand the knowledge of the human G ECM established previously with more matrix proteins identified showing even greater complexity of composition (139, 167). In addition, comparing the 178 ECM proteins (14 TI-only proteins + 126 shared proteins + 38 G-only proteins) in the ECM of intact tissue generated by combining the TI and G ECM proteins (Chapter 3) with the 91 ECM proteins identified in intact tissue samples without separation of G and TI (Chapter 4), a group of 85 matrix proteins were present in both. This result suggests that these matrix proteins might represent the higher
abundance proteins and analysis of individual tissue compartments (Chapter 3) enhanced the sensitivity of detection (potentially by enriching low abundance proteins). Overall, the data from proteomic analyses provide the baseline for future studies of pathological changes in the human kidney matrix. In addition to comparing the TI and G ECM, our study also compared young (<40 years old) and aged (>60 years old) kidneys to examine age-related changes in the ECM of the human kidney cortex. This analysis revealed age-related regulation of 8 proteins in the TI matrix all of which were up-regulated with age. This study also showed that 20 proteins altered with age in the G matrix (17 were up-regulated and 3 down-regulated). There were a relatively small number of proteins what appear to be altered with age which might due at least in part to the wide age range used in the young groups (15 to 35 years old).

There are some potential limitations in the proteomic analysis of the TI and G ECM (Chapter 3). Firstly, the use of enrichment technique, which is equivalent to a semi-decellularisation procedure, means that cellular components are not completely removed. Therefore, it is necessary to use ECM-defining databases to identify the matrix proteins, and different databases will return slightly different information based on the information contained in the particular database. Comparison of 3 different databases (HMDB, UniProt and GOTERM) for protein identification (Chapter 4, Section 4.4) showed that the HMDB provided the best coverage for ECM proteins and was, therefore, selected for matrix protein identification (Chapter 3 and Chapter 4). This leads to the second potential limitation of the study, i.e. the use of databases. Although matrix-defining databases are vital tools to identify ECM proteins in whole tissue samples, study of the decellularised human kidney cortex which lacks any cellular components and thus must contain primarily if not exclusively ECM/ECM-associated proteins. This study showed that when ECM-defining databases were applied to the MS data from the decellularised matrix, there was a marked reduction in the numbers of proteins suggesting that the database significantly underestimates the complexity of the composition of the matrix. For example, MS identified 478 proteins in the decellularised ECM, but when the HMDB was applied
to these data only 63 ECM proteins were identified in the decellularised samples (Chapter 4, Section 4.3.4). It should be acknowledged that some of the proteins in the decellularised kidney may be regarded as cellular in origin in that they represent proteins involved the cell adhesion to the matrix which may have remained associated with the ECM when cells were disrupted, e.g. integrins. Interestingly, Naba et al. (157) created the HMDB using bioinformatic techniques based on protein identification in UniProt in combination with the InterPro and SMART databases which contain information on protein structures. The entire human genome was screened to determine all hypothetical proteins which contain domains characteristic of ECM proteins, domains of proteins which can/may interact with or remodel the matrix as well as domains for signalling peptides (2, 157). By these criteria, proteins which contain uncharacterised matrix domains, novel matrix interaction domains or interact with the matrix through other proteins/protein complexes will not be represented in the HMDB. In a subsequent study, Naba and colleagues used the mouse matrisome database, which was also created using bioinformatic techniques, in combination with ECM enrichment to study the matrix of mouse pancreatic islets (154). Initially, 4097 proteins were detected in the enriched samples by MS, however, when these were filtered through the database 160 proteins were matched and identified as ECM proteins (154) suggesting that here too the application of the matrisome database restricted protein identification. Similarly, our study using enrichment of human TI and G ECM identified a total of 2047 proteins (Chapter 3), but when these proteins were put through HMDB, only 178 proteins were returned as ECM proteins. These examples show that although the HMDB is the most comprehensive matrix protein database (Chapter 4, Section 4.4), it may underestimate the number of proteins in the ECM. Based on our observations in the decellularisation study (Chapter 4) and published data, there is the potential to create a database using data on matrix proteins from decellularisation studies rather than bioinformatic data, which could be extended as additional studies are performed. A third potential limitation is that although our study is the first to provide protein profiling data on age-related
changes in the human kidney cortical ECM, the age range of the young group was relatively wide (15-35 years old) as only a small number of younger donor kidneys become available for research. It was considered preferable to have a wider age range with a higher number of samples than to only have a single sample in the young group.

The complexity of the kidney ECM is conferred only by the composition but also by the ultrastructure (12, 466). Within the kidney cortex, there are distinct regions of matrix, the glomerulus contains the mesangial matrix, the highly specialised GBM and Bowman’s capsule while within the tubulointerstitial matrix there are the tubular and capillary basement membranes, the matrices of larger vessel walls and the interstitial matrix (466). Therefore, it is of interest to understand how those different ECM compartments might regulate cell behaviour to provide insights into normal homeostatic mechanisms and better understand the changes that occur in disease. One way to study the effects of different matrix structures is to create a scaffold that retains both the structure and composition of the matrices which can then be recellularised. The majority of decellularisation studies have used kidneys from rodents and pigs with only a small number of studies focused on the human kidney (244). In the present study (Chapter 4), a protocol was developed to decellularise cubes of human kidney cortex tissue. The cube approach rather than decellularisation of the whole organ, was adopted for the following reasons: i) use of tissue cubes requires a relatively small amount of tissue which means that individual kidneys can be used for multiple purposes thereby maximising the use of individual organs; and, ii) samples from the same kidneys can be used to compare different experimental conditions, e.g. the effect of ECM scaffolds from the same kidney to support growth of different cell types, thereby reducing the number of experimental variables. Optimisation of the decellularisation protocol compared ionic and non-ionic detergents (SDS and Triton X-100, respectively) individually and in combination as well as different numbers and duration of detergent treatments and water washes. The final protocol involved multiple changes of SDS combined with water washes followed by a DNase treatment step. A variety of analyses showed that the resulting cubes were
acellular, contained minimal residual DNA and retained defined ECM ultrastructural features such as basement membranes (Chapter 4, Section 4.3.3). Previously published protocols for decellularisation of human kidney cubes of similar size took from 28 hours to 7.5 days (168, 235). Although our protocol used similar detergents and enzymes as these protocols, the length of time required for complete decellularisation was significantly reduced (~14 hours depending on the number of scaffolds processed) by introducing multiple chances of detergent. In addition, as discussed in Chapter 4, Section 4.4, our optimised protocol managed to reduce the exposure of matrix to SDS significantly, which could be beneficial to ECM preservation.

Proteomic analysis to compare the intact cortex and decellularised tissue from the same human kidneys (total 6 human kidneys used; ECM and soluble fractions were combined for analysis; Chapter 4 section 4.3.4) identified a total of 1082 proteins in the intact tissue and 478 proteins in the decellularised scaffold suggesting that the decellularisation procedure efficiently removed a significant proportion of proteins. These proteins likely represent the cellular proteins as histological sections showed complete removal of the cells, although the loss of matrix proteins as a result of the decellularisation procedure per se cannot be excluded. Interestingly the number of proteins in the human decellularised kidney scaffold in this study, is similar to a previous analysis of decellularised Rhesus monkey kidney slices in which 439 proteins were identified (232), although in this study there was no comparison with the ECM of the intact tissue. In addition to analysing decellularised monkey kidney matrix, Nakayama and colleagues analysed decellularised monkey lung slices and identified 282 proteins with less than half of these proteins (110) also detected in the decellularised kidney scaffold (232) suggesting that composition of lung and kidney matrices from the same species are distinct. Similar to the comparison made in our study, Li and colleagues compared decellularised whole human lung with intact tissue and identified 384 proteins in the decellularised samples while 2147 proteins were identified in the intact tissue (467). While this might suggest that the decellularised human lung matrix is more complex (i.e.
a greater number of proteins) than the monkey lung matrix, it is difficult to make this comparison directly as full list of identified proteins is not reported, and the difference in the number of proteins might also be attributed to species differences, differences in the decellularisation protocol and in the analysis. There are a limited number of human tissue decellularisation studies which also include proteomic analysis of the decellularised scaffolds: Nagao and colleagues reported 29 ECM proteins in decellularised cubes of human kidney cortex (168) while Leuning and colleagues identified 92 ECM proteins in the matrix of decellularised whole human kidney (162). Using MatrixDB (curated based on HMDB), Sackett and colleagues identified 120 matrisome proteins in decellularised cubes of human pancreas (468). Interestingly, recent reports of proteomic analysis of decellularised human liver identified relatively few matrix proteins with Verstegen et al. identifying 35 matrix proteins (469) and Mazza et al. reporting only 20 matrix proteins (470). All these studies used matrix-defining databases such as the HMDB, MatrixDB and GO terms ‘extracellular regions’, and did not provide either a full list of the proteins identified by MS or details of the databases used, therefore, direct comparison with our total protein data is not possible. Application of the HMDB to our data identified 63 ECM proteins in the decellularised scaffold compared to 91 ECM proteins in the intact tissue. The majority of proteins (58/63) in the decellularised scaffold were also present in the intact kidney ECM suggesting a core of matrix proteins are preserved after decellularisation. Among this core of matrix proteins, 44 out of 58 proteins are core matrisome proteins categorised by HMDB, including collagens, proteoglycans and glycoproteins, suggesting that these proteins might be more resistant to decellularisation treatment or strongly associated with matrix. The majority (20/33) of the proteins that were not detected in decellularised scaffolds but are present in the intact tissue ECM are ECM-affiliated proteins and ECM regulators which might be expected to be more loosely associated with the matrix. Although there was some loss of proteins during decellularisation it is clear that the human kidney ECM scaffolds retain a complex, multi-component matrix with distinct ultrastructural features which are absent from the gel-
based matrices often used to study the effect of ECM on renal cell behaviour and function (168, 244) thus, these scaffolds provide a novel platform for this type of study.

Having developed a decellularisation protocol for human kidney cortex tissue cubes and characterised the decellularised scaffolds, experiments were performed to examine how different renal cell types seeded on to this multi-component matrix. As mentioned earlier, an important advantage of cube methodology over the whole organ is that it allows a direct comparison of the effect of matrix derived from the same kidney on different cell types and also the comparison of the same cell types on scaffolds derived from different kidneys at the same time. This study compared the behaviour of each cell type on scaffolds from three different kidneys and showed that scaffolds from different kidneys do not have any markedly different effects on cell behaviour. Initial studies compared different seeding methods, including the number of cells reseeded per cube and the length of incubation of the reseeded cubes. The results showed that surface seeding of $4 \times 10^5$ cells/cube and incubation for 2 weeks post-seeding achieved the most extensive repopulation for all cell types. In the published data, the only human cell types used to repopulate decellularised human kidney matrix have been human endothelial cells and stem cells. These studies showed that endothelial cell repopulated the capillary structures while stem cells repopulated a variety of different structures including vascular- and tubule-like structures, and expressed different markers dependent on the localisation (162, 168, 235). In our study, three human renal cell types were selected as representative cell populations in the kidney cortex: proximal tubular epithelial cells (HK2) (the predominant epithelial cell type in the tubulointerstitium), podocytes (a key cell type in the glomerulus and in the control of blood filtration) and interstitial fibroblasts (a key cell type in maintaining normal kidney structure and which play an important role in fibrosis in multiple kidney diseases). Each individual cell type was seeded onto decellularised human kidney matrix scaffolds from the same 3 kidneys (Chapter 5). Similar to data reported for adult human renal stem cells seeded on human kidney matrix (235), HK2 were observed to align along tubular structures forming a monolayer of cells
around a lumen. Compared to glass, the matrix reduced proliferation (Ki67 staining) suggesting that the decellularised scaffold was able to slow proliferation of an immortalised cell line even in the presence of serum. Growth on the scaffolds also enhanced expression of differentiated proximal tubular epithelial cell markers LTL and AQP1, although expression of some mesenchymal markers persisted indicating that although the matrix differentiation it cannot drive full differentiation of HK2 cells. There was also a slight increase in apoptosis (cleaved caspase-3) which might indicate that the cells which could not differentiate die. In addition, other investigators showed that human endothelial cells seeded onto a human kidney matrix hydrogel also showed increased expression of the endothelial marker CD31 (162, 168) which is consistent with our result suggesting that the ECM could drive cell differentiation. However, in these studies, only matrices/gels were used, and there was no comparison to the more commonly used tissue culture substrate. Therefore, it was not possible to determine whether this was the benefit to endothelial cell growth compared to other substrates (162, 168). In our study, all three cell types showed decreased proliferation (Ki67 expression), enhanced differentiation and slightly increased apoptosis (cleaved caspase 3). Although the ECM reduced proliferation, the studies with the conditionally-immortalised podocytes illustrated the importance of the proliferative capacity of the cells at the time of seeding in that only undifferentiated podocytes (conditionally-immortalised cells grown at the permissive temperature and therefore proliferating) attached and repopulated the matrix while differentiated cells (grown at 37°C for 2 weeks prior to seeding on the scaffold) were unable to repopulate the scaffold. Similar to the proximal tubular epithelial cells, podocytes formed a monolayer of cells lining large lumens while in tubular profiles with small lumens the cells appeared as form clusters, filling the lumen. There was no indication of preferential localisation of podocytes to glomeruli possibly due to the lack of accessibility of intact glomerular structure. The expression of differentiation markers, synaptopodin and WT1, were increased in cells on the scaffolds although differentiation appeared to be incomplete as there was no change in the expression of nephrin (one of
the key differentiation markers for podocytes) in cells seeded on ECM versus on glass. However, it is not known whether these cells started to form foot processes. It may be speculated that the reduction in proliferation induced by the matrix is not enough and that further suppression of proliferation is required to achieve more complete differentiation. Similar to the conditionally immortalised podocytes, conditionally-immortalised fibroblasts also demonstrated a requirement for active proliferation at the time of seeding. The behaviours of interstitial fibroblasts on scaffolds were very different from that of tubular epithelial cells or podocytes in that they generally formed aggregates of cells in a few localised areas of the matrix although there were also some cells scattered through the ECM. These fibroblasts were seeded as a single cell suspension in the same way as tubular cells and podocytes, and it is not clear whether these foci of cells represent cells that migrated to form aggregates or proliferation of some cells which were localised to particular areas of the scaffold. The slight reduction in proliferation of fibroblasts on the scaffolds compared to glass at the end of the 2 week incubation period might argue against proliferation of cells to form foci, although a time-course of proliferation was not performed with proliferation measured only at the end of the incubation period. The fibroblasts showed increased expression of α-SMA-positive stress fibres suggesting enhanced myofibroblast differentiation which is characteristic of both normal wound healing and fibrosis (471, 472). Perhaps reflecting the increased myofibroblast (contractile) differentiation, the cells appeared to contract/remodel the matrix around the cell clusters. Similar contraction of the surrounding matrix was also observed in some individual fibroblasts scattered through the matrix. Contraction of the scaffold may also be due in part, to the physical configuration of the scaffold. During cell seeding, scaffolds were inserted into the individual wells of a round-bottom 96-well plate in order to maximise cell attachment with the matrix. However this can result in the scaffold forming a U-shaped matrix and seeding the cells into this fold such that cells are surrounded by matrix could promote cell adhesion and contraction of the matrix although this was not seen in either epithelial cells nor podocytes seeded onto matrices with the
same configuration however these cell types lack inherent contractility (473-475). In addition, the fact that the individual fibroblast, scattered in the matrix, appeared to contract the matrix around them might suggest that this can be an intrinsic response of this cell type to the decellularised matrix. It should be noted that kidneys used in this decellularisation study were greater than 60 years old and although the histology of these kidneys was described as “normal for age” by a renal pathologist some foci of fibrosis were present and matrix changes associated with fibrosis may promote cell contraction. It would be of interest to determine this behaviour is seen on scaffolds from younger kidneys. Another explanation might be that proteins that would attenuate the myofibroblast differentiation have been lost in decellularised matrix. It would also be of interest to determine whether this myofibroblastic phenotype can be of modulated by the presence of other cell types, e.g. epithelial cells.

With all cell types, the total area of the scaffold which cells repopulated was quite limited (~20%) and repopulation was generally confined to areas adjacent to the surface of the scaffold on which the cells were seeded. Individual scaffolds were quite large (5x5x2.5mm) and poor repopulation of the interior of the scaffold may reflect poor nutrient and gaseous exchange within the scaffold. Therefore, one aspect of future work (Section 6.2) would be to potentially reduce the size of the scaffolds and improve the culture conditions to enhance the repopulation of the scaffolds.

In conclusion, the work in this thesis provides the first detailed characterisation of the tubulointerstitial matrix of the human kidney cortex and has revealed both the similarities and differences in the TI and G matrices. Distinct age-related changes in the matrix of these cortical compartments were also identified. A relatively rapid decellularisation method was developed to produce sterile kidney cortex ECM scaffolds, and the decellularised matrix was characterised by microscopy and MS-based proteomics. These data highlighted the potential limitation of the current databases used to identify matrix proteins. Reseeding of the decellularised scaffolds with three representative human renal cortical cell types showed all three cell types could attach
and repopulate the matrix. Analysis of differentiation markers suggests that the matrix enhances the differentiation of epithelial cells and podocytes and may promote the myofibroblast differentiation of interstitial fibroblasts. Overall, this thesis has contributed substantial new knowledge of ECM of the human kidney cortex. Understanding the normal ECM and how this regulates cell behaviour is key to understanding the consequences of matrix changes in disease. Production and characterisation of decellularised human kidney ECM scaffolds and preliminary studies of the repopulation by different human renal cell types provides a platform to develop more complex in vitro models of the human kidney cortex which could be used to both the study how the ECM regulates cell behaviour and to create a novel in vitro human kidney model for drug screening.

6.2 Future work

There are a number of different aspects of the work presented in the thesis that it would be of interest to investigate:

- Similar to proteomics, mass spectrometry can be used to analyse global changes in non-protein matrix components, such as, polysaccharides (capillary electrophoresis–mass spectrometry), GAGs (reversed phase ion pairing LC/MS) and lipids (multi-dimensional mass spectrometry based shotgun lipidomics), in the ageing kidney ECM and in decellularised renal cortical scaffolds (476-478). An in-depth analysis of differences in non-protein ECM components in the different renal compartments with age would contribute additional information on the composition of the ECM in ageing and in disease.

- It would be of particular interest to characterise fibrotic human kidney cortex matrix using the same techniques as in Chapter 3 (separating G and TI compartments, depends on the level of fibrosis). At the moment, human kidneys for research become available through NHSBT and diseased kidneys are not generally available as they
are not suitable for transplantation. In general, failing kidneys are removed, however, it may be possible to collect diseased kidneys post mortem or, for example, where a transplanted kidney is removed following rejection (chronic allograft rejection). Comparison with our data on normal human kidney cortex matrix would enhance our understanding of how the human kidney matrix changes in fibrosis and potentially identify key changes that might be amenable to therapeutic intervention.

- Availability of fibrotic kidneys would also provide an opportunity to isolate cells from these kidneys for comparison with normal cells. The decellularisation protocol (Chapter 4) could be optimised for decellularisation of fibrotic kidneys and the decellularised scaffolds could be analysed as described for the normal kidney. These scaffolds could then be used to examine the effect of fibrotic scaffolds on the phenotype and function of different human renal cell types compared to normal ECM scaffolds. This comparison would provide insights into matrix-induced changes in fibrosis and identify potential therapeutic targets to intervene in the fibrotic process. The availability of normal and fibrotic kidney ECM scaffolds together with cells from normal and fibrotic kidneys would provide an exciting opportunity to assess the role of the matrix in driving pathological changes (normal cells on fibrotic ECM) or ameliorating pathological changes (fibrotic cells on normal ECM).

- Currently, decellularised scaffolds (Chapter 4 and 5) are used immediately after produced, but it would be important to ascertain whether they can be stored for future and/or shared use. Scaffolds could be stored either i) Refrigerated: at 4°C; or ii) Frozen. In this method, it is important to determine how different freezing method could affect the ultrastructure of the matrix such as snap-frozen, slow frozen. Cryoprotective chemicals, such as dimethyl sulfoxide (DMSO) and glycerol, should also be tested. However, it can be difficult to remove these chemicals from the scaffold. High-resolution microscopy, such as SEM and TEM, and proteomic analysis would be used to examine the changes in ultrastructure and composition of stored scaffolds over time.
- Repopulation of decellularised human kidney scaffolds with cells seeded on the surface of the scaffolds (Chapter 5) showed that cells only repopulate areas close to the surface of the cubes potentially reflecting limited gas exchange and nutrient supply within the scaffold. Future experiments would aim to improve the culture system. Three modifications that could be tested are:

  i) Reducing the size of the scaffold. The preliminary study suggests that scaffolds less than 1mm thickness were extremely difficult to handle and easily torn. Another approach might be the look at the feasibility of freezing the scaffold using cryoprotection reagents. Once a method for freezing scaffolds has been determined, it might be feasible to section the frozen scaffolds to create scaffolds (~100 µm), and the sections supported on a mesh at the medium-air interface. These scaffolds could be used in combination with a modified perfusion method such as organ-on-chip (Elveflow) to enhance repopulation.

  ii) Incubating the reseeded cubes in a multi-well plate or petri dish with constant perfusion of fresh medium using a perfusion insert for a petri dish (PCP-1, Automate Scientific). The level of pH and oxygen inside the scaffold would be measured using fibre optic pH and oxygen microsensors and could be controlled to mimic intact tissue (pO₂=7%) (502123 and 501656, World Precision). However, the exchange of medium (nutrient and gas) inside the scaffold is still dependent on free diffusion.

  iii) The decellularised matrix scaffold could be placed on a structure similar to a Trans-well insert (modified using commercially-available inserts such as Netwells inserts (Corning)) and with medium to pass through the scaffold by gravity and exiting at the bottom of the insert. The fresh medium could be placed on the top. However, a disadvantage of this system would be that the medium needs to be changed manually. In optimising the culture system, a
variety of variables need to be considered depending on the objective of the experiments, such as ease of handling, and the possibility for high throughput.

- The kidney contains ~26 different cell types with important heterotypic cell interactions. The ultimate goal is to create normal and fibrotic tissue mimetics containing multiple cell types within an ECM scaffold. In order to achieve this, multiple cells could be seeded onto the scaffolds and tracked with different commercially-available fluorescence cell tracker dyes (may require labelling before seeding) (Thermo Fisher) in combination with 3D imaging by confocal to determine whether the seeding of multiple cell types affects the distribution of cells compared with seeding each cell type individually. Changes in proliferation, apoptosis and differentiation would also be assessed. The generation of a human multi-cell type tissue mimetic would provide a novel platform for drug screening. Clearly, development of complex tissue mimetics would require optimisation of wide number of variables including, cell types for seeding (from cell line to primary cells), sequence of seeding different cells types (which cell types should be seeded first), ratio of different cell types (different ratio of number of cells in different tissue compartments), location and composition of settled cells (in an area of settled cells, whether there is a single cell type or a mixture of different cell types when cells settled on the matrix) and function of settled cells (expression of key function markers for this cell types in normal kidney).

- The decellularised scaffolds have definite advantages in retaining the endogenous ultrastructure, however, there are some technical challenges in using these scaffolds, and it may be that at least some of the advantages of providing cells with a kidney ECM could be achieved by using a hydrogel (168, 211). In addition to optimising the cell-decellularised scaffold culture system, a human kidney hydrogel could be developed. Using a hydrogel, it is possible to look at the effect of changing the mechanical property of matrix such as stiffness and deformability on cell behaviour, e.g. morphology of mouse embryonic fibroblasts (BALB/c 3T3) was affected by
different stiffness of the matrix (479, 480). However, the limitation of this approach is that the structure of the matrix is lost, and all the matrix components homogenised. More recently, the introduction of 3D printing has provided a means to create scaffolds with some structural details (195, 481) which could be applied to hydrogels although these would lack the specialised composition of particular ultrastructural features, e.g. tubular basement membranes, and limitations on precision and gelation of 3D printing would preclude creation of complex structures such as glomeruli. Although 3D printing is currently somewhat limited, there are likely to be technological advances that are will allow construction of more complex matrices.

As outlined above, there are a number of interesting aspects to the work which could be explored, in particular, the creation of a multi-cell type tissue mimetic would be important both for understanding the role of the ECM in normal kidney homeostasis and disease and in providing a human cell-based platform for drug testing.
Appendix

Please see the attached CD for:

Appendix Table 2.1 List of unique peptide sequences for collagen isoforms identified by MS.

Appendix Table 3.1 List of ECM Proteins identified in the human tubulointerstitial (TI) matrix.

Appendix Table 3.2 List of ECM Proteins identified in the human glomerular matrix.

Appendix Table 3.3 Novelty of TI ECM proteins identified by UniProt and Pubmed databases.

Appendix Table 3.4 List of ECM proteins only identified in the human glomerular matrix

Appendix Table 4.1 List of proteins in the decellularised human kidney cortical samples identified by proteomics.

Appendix Table 4.2 List of proteins in the un-decellularised (intact) human kidney cortical samples identified by proteomics.
Reference

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