KIDNEY TISSUE ENGINEERING USING DECELLULARIZED ORGANS AND PLURIPOTENT STEM CELLS

By

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To Jennifer, Connor, and Caden

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LIST OF ABBREVIATIONS

AFS CELLS	Amniotic fluid stem cells
BM	Basement membrane
BMP	Bone morphogenetic protein
BRDU	Bromodeoxyuridine
BrdU	Bromodeoxyuridine
BSLB4	*Bandeiraea simplicifolia* lectin B_4
BUN	Blood urea nitrogen
CC3	Cleaved caspace-3
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CK	Cytokeratin
CMA	Cranial mesenteric artery
CMC	Critical micelle concentration
DAB	3,3'-Diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNASE	Deoxyribonuclease
EBS	Embryoid bodies
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPCS	Endothelial progenitor cells
ES CELLS	Embryonic stem cells
ESRD	End stage renal disease

FBS	Fetal bovine serum
GBM	Glomerular basement membrane
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescent protein
GSG	Glycosaminoglycan
H&E	Hematoxylin and eosin
HGF	Hepatocyte growth factor
HSPG	Heparan sulfated proteoglycan
HUVECs	Human umbilical vein endothelial cells
IHC	Immunohistochemistry
IMDM	Iscove's modified Dulbecco's medium
iPSCs	Induced pluripotent stem cells
iRAD	Implantable Renal Assist Device
LIF	Leukemia inhibitory factor
mES cells	Murine embryonic stem cells
MET	Mesenchymal-epithelial transition
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MSCs	Multipotent stromal cells
NaDC	Sodium deoxycholate
PBS	Phosphate buffered saline
PCL	polycaprolactone
PECAM-1	Platelet endothelial cell adhesion molecule
PLGA	poly(lactic-co-glycolic acid)
PMSF	Phenylmethylsulfonyl fluoride

QRT-PCR	Quantitative reverse transcriptase polymer chain reaction
RAD	Renal Assist Device
RET	Receptor tyrosine kinase
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TBS-T	Tris-buffered saline + 0.1% Tween
TEM	Transmission electron microscopy
U-NAG	Urinary N-acetyl-beta-D-glucosaminidase
VBM	Vascular basement membrane
VIWAK	Vicenza wearable artificial kidney
WAK	Wearable artificial kidney
XNAS	Xenoreactive natural antibodies

Abstract of Dissertation Presented to the Graduate School
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KIDNEY TISSUE ENGINEERING USING DECELLULARIZED ORGANS AND PLURIPOTENT STEM CELLS

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Each year, there is a severe shortage of kidneys for organ transplant. One approach to alleviate this demand is to reconstruct kidneys using tissue engineering scaffolds. Such scaffolds may be produced through the decellularization of whole organs. Ideally, the resulting extracellular matrices would retain the organ-specific architecture and chemical composition to guide implanted cells into functional structures and eventually working organs. Pluripotent embryonic stem cells are highly proliferative, capable of differentiating into all cells of the kidney, and respond to tissue-specific extracellular matrix proteins. Examining the ability of these cells to proliferate and differentiate in a whole kidney extracellular matrix scaffold would serve as a valuable baseline for future studies in producing a functioning organ.

Decellularization of whole rat kidneys was achieved by perfusion of detergent-based solutions through the renal artery. The most effective 5-day protocol utilized Triton X-100, sodium dodecyl sulfate (SDS), salts, and deoxyribonuclease (DNase) and was determined by modifying detergent concentration and perfusion duration and testing an alternative ionic detergent. The resultant organ-shaped structure was spongy and translucent. Hematoxylin and eosin histochemical analysis and electron microscopy showed preserved glomerular, vascular and tubular structures without observed nuclear material and only trace amounts of residual

cellular debris. In addition, immunohistochemistry (IHC) showed removal of cellular proteins and preservation of essential basement membrane (BM) proteins collagen IV and laminin.

Murine embryonic stem cells were seeded into the decellularized scaffolds and cultured up to 14 days with one of three protocols: whole scaffold, perfused scaffold, and sectioned scaffold culture. Cells delivered into the renal artery showed initial glomerular localization with subsequent expansion into adjacent vasculature, interstitium, and tubules. Over time, histological analysis revealed distinct cell morphologies, patterns of cell division and apoptosis, and cell arrangements with Pax-2 and cytokeratin positivity. Cytokeratin is an epithelial cell marker and Pax-2 is necessary for kidney development. The evidence of cellular change of mouse stem cells in response to a rat scaffold is a promising step toward establishment of a xenogenic scaffold source for engineered kidneys.

CHAPTER 1
INTRODUCTION

Once an organ such as the kidney reaches a state of irreversible degradation, organ function must be replaced to ensure survival of the patient. Currently, this is best accomplished through organ transplantation. In the United States, as of November 16, 2012, a total of 116,447 people were candidates for organ transplant, and of those, 81% were waiting for kidney transplantation. However, donors are in short supply. In 2011, only 16,800 candidates received a transplanted kidney (Based on OPTN data as of November 16, 2012). Renal replacement therapies such as hemodialysis and peritoneal dialysis extend life expectancy and may bridge the time gap before transplantable organs becomes available, but at the cost of significant associated complications and increased rate of mortality over time.[1] Advancements in these technologies and associated treatment strategies steadily improve patient outcome, but they are still not adequate replacements for a functioning organ. Fortunately, advances in the new field of regenerative medicine give hope that alternate sources of renal tissue may be created.

A definition of regenerative medicine as given in an editorial by Mason and Dunnill (2008) is "regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function." Approaches typically include cell-based therapies with optional gene modification, biomaterials, and methods from molecular medicine. Stem cells are likely to play a key role.[2] One such approach to address the kidney shortage is to use a tissue scaffold to guide implanted cells into a functional replacement organ. However the kidney is a complex organ with over 30 cell types arranged in intricate structures.[3] It is the precise arrangement of these cells on the renal extracellular matrix (ECM) that allows the kidney to perform its critical functions. These functions include blood filtration, waste removal, electrolyte regulation, blood

volume control, and stimulation of red blood cell production.[4] Anthony Atala stated that renal tissue is "arguably one of the most difficult tissues to replicate in the laboratory." [5]

Current techniques of fabricating cellular scaffolds include electrospinning, which has been used to create meshes of biopolymer with extracellular matrix proteins or bound bioactive peptides, and gas-based techniques, which generate biomaterials with interconnecting pores for improved nutrient diffusion and cellular waste removal.[6-9] Although noteworthy, these techniques cannot yet mimic the advanced histoarchitecture of native renal ECM, and are unlikely to promote functional arrangement of seeded cells. Extracellular matrix is a three dimensional network comprised of proteins, proteoglycans, and glycoproteins that supports cell attachment and arrangement, acts as a regulator and reservoir for growth factors, and modulates cell signaling. Kidney ECM is crucial to renal function as it is a primary component in glomerular filtration and supports each segment of distinct epithelial cells with unique underlying matrix composition.[10] The extraction of ECM from a kidney may provide a sufficient scaffold for seeded cells to form renal structures and eventually a whole functional kidney; this can be accomplished with a process called decellularization.

Decellularization is the process that removes cells from tissue leaving extracellular matrix. Methods for decellularization include mechanical agitation and shearing, freeze thawing, sonication, and washing with acid or detergent. The overall goal is to successfully remove any immunogenic cell remnants while preserving beneficial extracellular matrix proteins.[11] Extracellular matrices from both human and porcine sources have already been implanted clinically with or without growth factors to treat a variety of conditions. These scaffolds have been derived from bone, skin, bladder, nerve, heart valve, and small intestine.[12-17] They typically rely on influx and differentiation of host cells to effect repair. Engineering a functional kidney,

however, may require intact whole organ ECM structure with population and arrangement of the multiple cell types *in vitro*. Extracellular matrices for the purpose of organ reconstruction have been prepared from whole heart, lung, liver, and pancreas.[18-27] In most of these cases, the primary method of decellularization is perfusion of detergents through the vasculature.[28]

A promising cell source for scaffold-based kidney generation is pluripotent stem cells.[29] These cells have a high capacity for proliferation and the potential to differentiate into all cell types in the kidney. In addition, extracellular matrix isolated from a tissue has been shown to influence differentiation of pluripotent cells toward that tissue's cell lineages.[20, 30] Pluripotent cell lines have been established from embryonic stem cells, amniotic fluid stem cells, and induced pluripotent stem cells.[31-33] Other potential cell sources such as adult multipotent stem cells have been identified in adult kidneys, but they have not been shown to account for all cell types in the kidney.[34-37] Extrarenal stem cell sources such as hematopoietic stem cells, endothelial stem cells, and multipotent stromal cells (MSCs) are another option; however, given testing on current injury repair models, they have not readily contributed to nephrogenic cell lineages.[38, 39]

The hypothesis for this research is that an extracellular matrix tissue scaffold can be produced with perfusion-based organ decellularization, and with unique renal-specific histoarchitecture best support the multitude of cell types necessary for proper kidney function and ultimately the generation of a replacement organ. While proving this hypothesis will likely take decades of research, the testing of two specific aims will provide fundamental knowledge for advancement of kidney organ engineering. The first will determine if cells and cellular debris may be removed from a whole rat kidney while preserving extracellular structures and matrix

proteins. The second will test the ability of the ECM scaffold to effect attachment, proliferation and differentiation of injected pluripotent murine embryonic stem cells.

The development of a reliable process for kidney-specific whole organ scaffold production would be a significant step toward the eventual generation of a new organ. Such a scaffold would allow the *in vitro* examination of cells in the outline of functional structures and in contact with retained extracellular molecules. Optimal conditions for cell growth and site-specific differentiation may eventually be interpreted. Testing the response of pluripotent stem cells to the scaffold will serve as a baseline for further studies in scaffold preparation and modification, cell source alternatives, and culture variations with the goal of functional structure formation. As the removal of cellular debris removes a significant component of destructive host response, this research may lead to new organs formed from non-donor matched kidneys, kidneys with cellular disease yet with preserved matrix, or xenogenic sources.[40-42] Additionally, pluripotent stem cells can now be derived from patient-specific, non-invasive cell sources, so manipulation of these cells into functional structures may be clinically relevant.[43, 44]

This dissertation is divided into five additional chapters including a survey of relevant research, development of whole kidney decellularization, response of embryonic stem cells to renal extracellular matrix scaffolds, an exploration of options for further research, and details of the procedures used in this research. Appendices include further details of the constructed apparatuses utilized in these studies and supplementary histology images.

CHAPTER 2
BACKGROUND

End Stage Renal Disease and Renal Failure

One of the most prominent healthcare concerns in the country is renal failure, or the inability of the kidney to remove wastes and maintain electrolyte balance. The non-reversible condition that will inevitably lead to kidney failure is end stage renal disease (ESRD). Acute renal failure and chronic kidney disease can both progress to ESRD. In 2010, almost 600,000 people were treated for ESRD in the U.S., with associated healthcare costs estimated at $47.5 billion. A majority of ESRD patients have a primary diagnosis of hypertension and/or diabetes. The treatment for ESRD is renal replacement therapy, mainly hemodialysis, peritoneal dialysis, and ultimately kidney transplant. Dialysis treatments are used to extend life until a transplantable kidney is obtained, but they are costly, and the 5 year survival rate for those on dialysis is only 35%. Kidney transplant markedly improves long term mortality, with 5 year survival improved to 73%, but the severe shortage of procured organs considerably limits availability of this treatment option.[1] In 2012, November, 94,556 people were waiting for a kidney transplant, yet in 2011 only 16,813 patients received transplants (Based on OPTN data as of November 16, 2012). Production of functional, implantable tissue engineered kidneys would significantly impact the population of transplant candidates. Furthermore, as the estimated primary cause of long term graft failure is antibody-mediated rejection, a kidney designed to be non-immunogenic would improve long term survivability of transplant recipients.[45]

The Kidney

Knowledge of renal structure and function, including natural extracellular architecture, organ development, and response to injury will contribute to the creation of functional kidney tissue.

Anatomy and Physiology

The kidney is a complex bean-shaped organ that filters blood, removes waste, balances electrolytes, mediates acid-base balance, controls blood volume and stimulates red blood cell production. It contains primary functional units called nephrons. Each nephron contains a renal corpuscle which sits at the head of a multi-segment uriniferous tubule. The renal corpuscle contains a capillary tuft, the glomerulus, surrounded by a spherical chamber, the Bowman's capsule. Almost 25% of the blood pumped from the heart enters the kidney through the renal artery and then proceeds through branching arteries to the glomerulus. Blood filtrate passes through the fenestrated endothelium of the glomerulus, the glomerular basement membrane, and visceral epithelial cells or podocytes. The Bowman's capsule then captures the filtrate and directs it down the tubule. The blood continues to move through a peritubular capillary network before returning to circulation through the venous system. In the tubule, a sequence of segment-specific, cell-mediated absorption and reclamation processes allow water and necessary metabolites such as glucose and amino acids to pass back into circulation through the adjacent peritubular capillary network. The remaining filtrate then passes through the collecting ducts, converges into the papillary ducts of Bellini, empties into the calyces, and departs through the ureters. The parenchyma of the kidney consists of two gross structures in radial alignment, the superficial cortex and deeper medulla. The medulla is further divided into an outer and inner zone. The cortex mainly contains the glomeruli and convoluted sections of the tubules. The medulla contains the straight portions of the tubules and collecting ducts.[4]

Extracellular Matrix

The extracellular matrix exists as a network of proteins and polysaccharides that is produced by and supports cells in a tissue. Specialized sheets of extracellular matrix called basement membranes underlie epithelial cells and connect them to the interstitial matrix.

Basement membranes are necessary for normal tissue function and development and defects cause diminished function. They also play a key role in glomerular filtration.[10] They regulate cell adhesion, polarity, migration, growth, and differentiation.[46] The adult kidney has distinct ECM composition within its vascular network, Bowman's capsule, glomerular basement membrane, proximal tubule, loop of Henle, distal tubule, collecting duct, and the mesangial matrix (Figure 2-1). Component proteins of the basement membrane include collagen IV, laminin, entactin/nidogen, and sulfated proteoglycans.

Collagen IV provides the covalently bonded latticework that structurally supports the basement membrane. The polypeptide chains which assemble to form a collagen IV triple helix are primarily distributed as follows: α1 and α2 in all renal basement membranes; α3, α4, and α5 in glomerular and distal tubular basement membranes; and α5 and α6 in Bowman's capsular, distal tubular, and collecting duct basement membranes.[10] Laminins are ubiquitous basement membrane glycoproteins which self-associate, form sheets, and commonly connect with Collagen IV through entactin/nidogen, perlecan, and fibronectin.[47, 48] Further, laminins act as the primary binding sites for cellular integrins and play a major role in cell attachment, differentiation, and preservation of phenotype; maintenance of tubular and vascular structures; and numerous nephrogenic processes.[10, 46, 49, 50] The isoforms of laminin differ with tissue type and developmental stage. The primary laminins in vascular basement membranes are 411, 421, 511, and 521, with 111, 211, 511, and 521 in the remainder of the adult kidney basement membranes.[10, 46]

Proteoglycans are found in all basement membranes, and consist of protein cores with the glycosaminoglycan side chains hyaluronan, heparan sulfate, chondroitin sulfate, and/or dermatan sulfate. These glycosaminoglycans stabilize the basement membrane through binding with

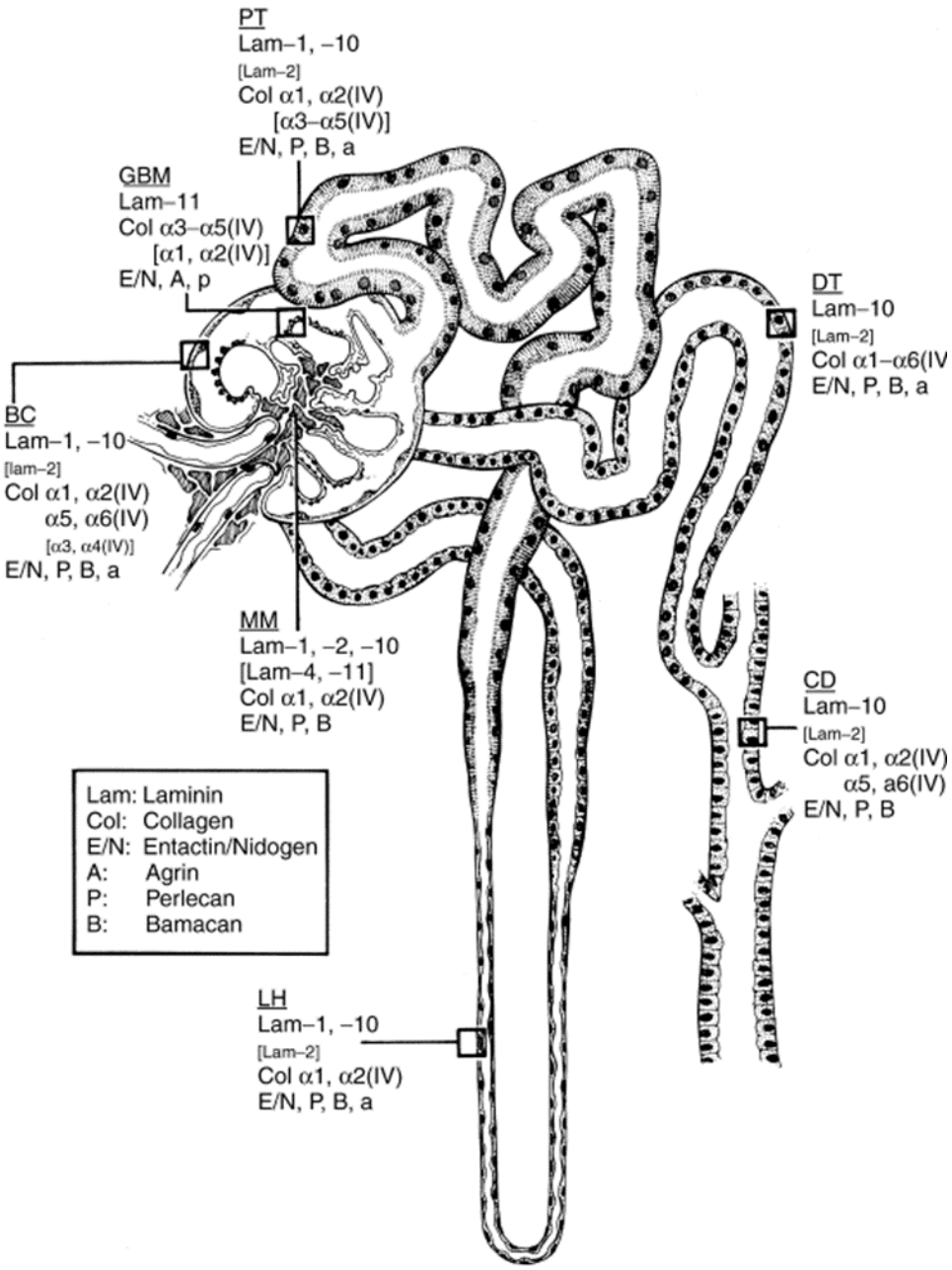

Figure 2-1. Basement membrane of the adult kidney nephron is a rich tapestry of proteins, divided into functional segments. BC, Bowman's capsule; CD, collecting duct; DT, distal tubule; GBM, glomerular basement membrane; LH, loop of Henle; MM, mesangial matrix; PT, proximal tubule. Reprinted by permission from Macmillan Publishers Ltd: Kidney International. Miner JH. Renal basement membrane components. vol. 56, pg. 2017, (Figure 1), copyright 1999.

laminin, collagen IV, and entactin/nidogen and are important mediators of cell behavior. They are necessary for growth and development in the kidney.[48, 51] Proteoglycans in the kidney include agrin, perlecan, bamacan, and collagen XVII.[10] Another function of proteoglycans is to bind and

regulate growth factor delivery.[48, 49, 52, 53] Growth factors such as VEGF, PDGF, HGF, FGF, TGF-β, BMP-7, and Wnt-4 are all involved in renal processes.[52, 54-56]

Renal extracellular matrix constituents play a role in development.[10, 51] There is a distinct spatiotemporal expression profile; at various stages of development, there are shifts in renal extracellular molecules, including alterations in collagen alpha chain and laminin isoform expression.[57] Change of normal extracellular matrix expression patterns can result in diminished or loss of function in the kidney.[10, 48, 51] For example, entactin/nidogen is required for tubulogenesis and collagen XVIII is required for ureteric bud branching.[48]

Organogenesis

The processes involved in transformation of embryonic cells into an adult organ can give insight into potential stem cell sources, tissue patterning, or the differentiation of stem cells into functional adult phenotypes.

The origin of kidney development can be traced back to the inner cell mass of the blastocyst; it forms a single sheet of cells called the epiblast. The cells of the epiblast, in the process of gastrulation, migrate along the primitive streak and develop into embryonic endoderm, mesoderm, and ectoderm. Mesodermal tissue then exhibits a patterning whereby the lateral axis segregates into intermediate and lateral plate mesoderm. From the intermediate mesoderm, the nephric or Wolffian ducts form, in which rudimentary pronephric and mesonephric epithelial kidney tubules branch.[58] However, in higher vertebrates, these structures are supplanted by metanephric kidney development, which is initiated on the caudal end of the nephric ducts (Figure 2-2). In this process, an outgrowth of the duct called the ureteric bud extends into metanephric mesenchyme and induces aggregation of cells around the tip.[59] This occurs at E11 (days after conception in mouse). These cells, known as cap mesenchyme, are considered stem cell precursors for all epithelial cells in the nephron except those in the

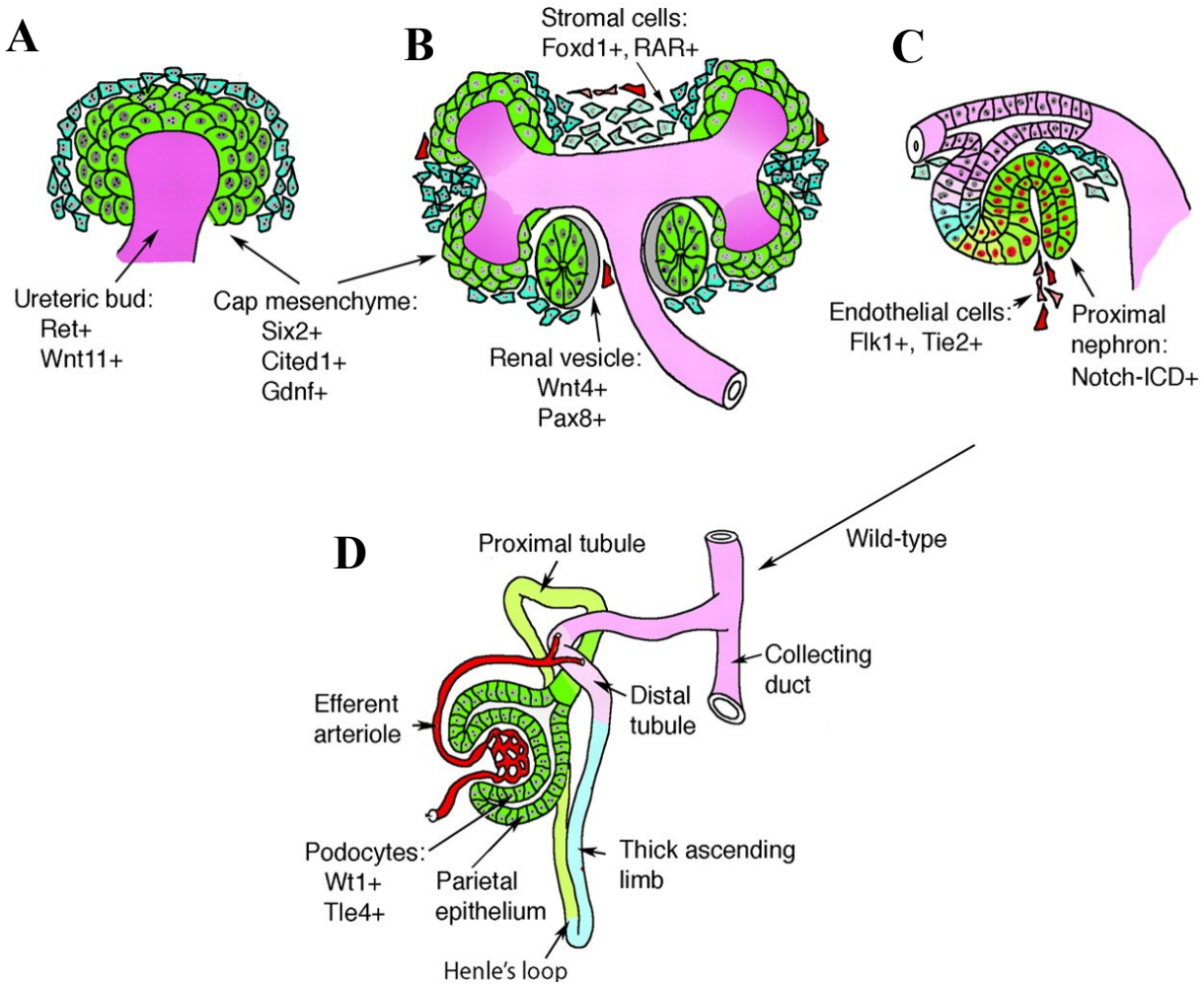

Figure 2-2. Development of the nephron. A) Branching ureteric buds extend into the metanephric mesenchyme, inducing condensation of the cells at the tip into cap mesenchyme. These are stem cell precursors to all epithelial cells in the nephron. B) Mesenchymal cells that do not aggregate form stromal cells that are precursors of vascular pericytes and cells in the interstitium and mesangium. Cap mesenchymal cells then polarize to form renal vesicles. C) These vesicles form S-shaped bodies, merge with the collecting system, recruit vasculature, and D) develop and elongate into mature nephrons. Key molecular markers expressed at each stage are indicated. Adapted with permission: Development. Dressler GR. Advances in early kidney specification, development and patterning. 2009; 136(23), pg. 3867, (Figure 4).

collecting duct.[60] Conversion of these cells into epithelium is known as mesenchymal–epithelial transition (MET). The signals generated during the formation of aggregates cause the bud to branch and extend more bud tips. Meanwhile, cap mesenchyme cells polarize to form renal vesicles, which cleft once to become comma-shaped-bodies, and again to form s-shaped bodies. One end of these s-shaped bodies connects back to the branching ureteric system and the other

recruits endothelial cells to form the glomerular capillaries. Over time, and through a series of intricate molecular interactions, the nephron matures and the cells differentiate into at least 26 different cell types (based on morphology, location, and function) associated with an adult kidney.[58, 61]

All cells in the adult kidney originate from the intermediate mesoderm. From that source, distinct transitional cell populations, identified by unique combinations of expressed molecular markers, progressively differentiate and interact over time to form the necessary adult somatic cell types. A basic lineage map summarizing the acknowledged renal cell populations and pathways of differentiation is given in Figure 2-3.[62] This map, with associated population markers, can be used to establish renal differentiation of stem cells. The Osr1 gene is the earliest known marker of intermediate mesoderm. It is required for the expression of other factors involved in kidney formation such as Eya1, Six2, Pax2, Sall1 and Gdnf (glial cell line-derived neurotrophic factor).[63] Lim1 (lhx1) is also expressed in the intermediate mesoderm, and later in the nephric duct, and many of the structures of the developing metanephros. All epithelial patterning in the intermediate mesoderm requires Lim1.[64] In addition to lim1 and odd1, pax2 and pax8 are also expressed in more mature intermediate mesoderm.[58, 65] Hox genes mediate mesodermal patterning; Hox11 is required to distinguish metanephric mesenchyme.[59] Two genes directly involved with regulating ureteric bud outgrowth are Gdnf, which is secreted by the metanephric mesenchyme, and its receptor, Ret (receptor tyrosine kinase), in the ureteric bud epithelium. Secretion of Wnt9b from the ureteric bud tip then initiates MET.[60] Condensed mesenchyme or cap mesenchyme is distinguished by Six2, Cited1, and Gdnf.[59, 60] The release of Wnt9b also upregulates Fgf8 and Wnt4, the latter activating renal vesicle formation and

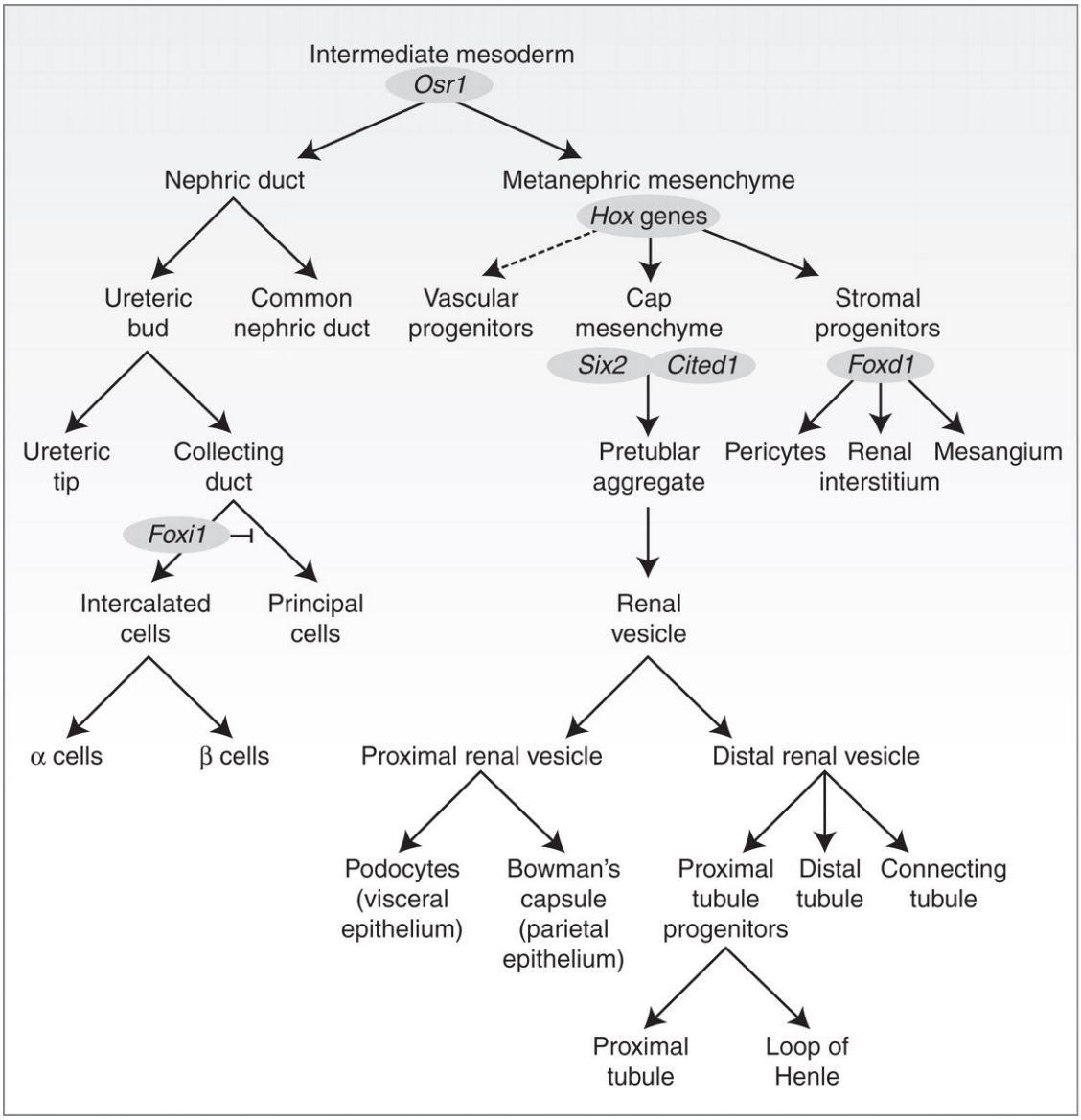

Figure 2-3. Renal developmental lineage map. Two primary cell populations, nephric duct and metanephric mesenchyme are derived from intermediate mesoderm. The nephric duct gives rise to the renal collecting system, whereas the metanephric mesenchyme is a precursor to all other cells in the nephron as well as vascular and stromal populations. Cells in the cap mesenchyme are a precursor to all epithelial cells in the nephron. Ovals indicate anchor marker genes, unique to the specific population. Reprinted by permission: Cold Spring Harb Perspect Biol 2012;4:a008300.

polarization of epithelia. Foxd1 identifies stromal progenitors such as interstitial or mesangial cells, also derived from the metanephric mesenchyme.[60]

Other significant genes involved in the molecular regulation of nephrogenesis include Pax-2, WT-1, Eya-1 and Sall1. Pax2, a critical factor in branching morphogenesis, is expressed

in ureteric bud, metanephric mesenchyme, and cap mesenchyme.[65-68] Pax2 is also necessary for selective differentiation of cap mesenchyme and Foxd1+ stromal stem cells into more mature phenotypes.[38] WT-1 is a transcription factor that represses Pax-2 during normal development, allowing terminal differentiation in the metanephros.[69, 70] Eya-1, a specifier of metanephric mesenchyme, plays a governing role in Gdnf regulation and is essential for nephrogenesis.[71] Sall1 is yet another gene required for ureteric bud invasion and induction of metanephric mesenchyme.[72] Many of the genes and proteins listed are also implicated in adult reparative pathways, such as Pax-2.[73]

Kidney Repair

The kidney has innate mechanisms for damage repair. Benigni *et al.* (2010) provide an informative summary, but a complete picture has not been established. Nevertheless, sources of repair have been reported to include differentiated renal cell types, extra-renal cells, and kidney stem cells. The primary contributor to replacement of apoptotic or denuded cells caused by ischemic injury has been shown to be adult renal epithelial cells.[38] One proposed mechanism is dedifferentiation, migration, proliferation and re-differentiation of the epithelial cells.[73, 74] Furthermore, it has been suggested that epithelial cells gain plasticity and revert to functionally active progenitors through epithelial to mesenchymal transition.[38] Extra-renal cells such as bone marrow derived stem cells, endothelial stem cells and mesenchymal stromal cells do not normally contribute to nephrogenic lineages.[39] However, they have been shown to have a supportive role in renal repair through mobilization to site of injury and paracrine signaling.[38] Resident epithelial cells and macrophages have also been shown to have a localized paracrine effect.[38, 75] For example, macrophages, which are adept at invading tissue, release Wnt7B into injured kidney tissue, promoting basement membrane repair, reduction of apoptosis, and tubule repopulation.[75]

Stem cells have been isolated from the adult kidney, but their role in repair has not been entirely elucidated. They have been shown to activate upon injury, enter into cell division, and mobilize to sites of repair.[39] They have also been shown to engraft into developing kidney cell populations, yet their strength may be as another supportive paracrine influence.[36, 37, 76] Suspected renal stem cells have been identified by expression of recognized stem cell markers, lack of differentiated cell markers, prolonged cellular quiescence, enhanced proliferation capacity, or mobilization upon injury.[34, 35, 37, 38] In addition, these cells are typically tested for ability to differentiate into renal epithelial cells, integrate into a developing kidney, and differentiate into unrelated cell types to affirm multipotent character.[34, 35, 37, 38] Adult kidney stem cells have been isolated from glomerular parietal epithelium, tubules, collecting ducts, papillae, and related interstitium.[35, 38, 74, 76, 77] One example, called label-retaining tubular cells, was identified using bromodeoxyuridine (BrdU) labeling. Peritoneal cells were exposed over time to BrdU, which incorporated into replicating cell DNA. When exposure was finished, rapidly dividing cells lost BrdU, leaving slowly dividing cells with retained label. These labelled cells integrated into developing metanephric kidneys, proliferated after ischemic injury, and responded to growth factors known to effect kidney repair.[36] These cells may correspond with cells identified by Gupta *et al.* (2006), which showed expression of stem cell (mesenchymal, embryonic, and developing kidney) markers vimentin, CD90, Oct4, and Pax-2, and lacked expression of differentiated cell markers MHC class 1 or 2 or cytokeratin.[34] Two other markers that have been used for kidney stem cell identification are CD24 and CD133.[74, 76, 77] Presence of CD24 and CD133 and absence of PDX, a marker of podocyte and endothelial cells, was used to identify the renal progenitors located in the urinary pole of the Bowman's capsule, adjacent to the tubule opening. These cells contributed to both the podocyte and tubule cell populations after

renal injury.[77] Unfortunately, these tissue-specific stem cells, as well as other innate mechanisms for kidney repair, are unable to regenerate lost tissue or compensate for effects of acute renal failure or chronic kidney disease.

Current Approaches to Renal Tissue Engineering

There are several proposed avenues to address the need for functional kidneys: the improvement of current transplant technology, the use of cellular engineering techniques to provide reparative cells to damaged tissue, generation of tissue rudiments, and construction of implantable bio-artificial renal structures.

Kidney Xenotransplantation

The primary concern in kidney transplantation is the limited number of viable donor organs. Therefore, xenotransplantation is being pursued as an alternative. Organs from other animal species, such as pig, would solve the demand, but the severe immune response resulting in graft rejection would need to be mitigated. The current state of kidney xenotransplantation is reviewed by Ramackers et al. (2012).[78] Transplantation of a xenogeneic organ provokes a hyperacute rejection response, which is caused from activation of xenoreactive natural antibodies (XNAs) in the host. These XNAs are present without immunization and target xenogenic antigens. The primary target of XNAs is alpha-gal epitope, which is expressed on all animals except human and humanoid primates. Identified targets of this and other XNAs may be resolved by gene-knockout in the donor tissue, but acute vascular rejection still remains a concern. Acute vascular rejection develops from an adverse interaction between human blood and xenogenic endothelium resulting in coagulation and subsequent graft failure. Progress has been made with transgenic expression of protective and anti-thrombotic proteins, but a viable xenogeneic transplant is still not likely in the near future. One avenue to mitigate rejection may be to implant primordial xenogeneic kidneys (renal metanephroi) and allow them to develop *in situ*, as

xenoreactive antigens are not present in renal tissue at an early stage of development. The vasculature can be recruited from the host, in essence removing a significant source of graft rejection. These experiments are in early stages however, and pig to primate implantation still needs to be performed.[78] In addition, other concerns such as viral transmission and determination if developed organs will meet physiologic demand need to be pursued.[79]

Cell-based Therapies

A popular strategy for the repair of tissue is to deliver cells that will replace damaged cells, stimulate localized repair, or even generate new tissue. Epithelial cells, adult kidney stem cells, extra-renal stem cells, and even amniotic fluid stem cells have all been used in this context. In one effort to provide a possible regenerative treatment for chronic kidney disease, a tubular cell-enriched population of primary renal cells embedded in a gelatin hydrogel was injected into a healthy rodent kidney. The cells showed evidence of neo-kidney tissue formation with structures resembling early glomeruli and tubules with little evidence of cell necrosis or destructive inflammatory or fibrotic response. However, long term cell integration and functional improvement have yet to be evaluated.[80]

Adult stem cells are linked to the body's normal maintenance and injury response pathways. Isolation and delivery or mobilization of these cells into the damaged area is a logical step in promoting kidney repair. In current models of kidney repair, the selected stem cells are usually delivered arterially or injected under the kidney capsule. Significant achievements include integration of cells into injured parenchymal tissue and expression of differentiated renal epithelial markers. Cells may also self-assemble into new structures. Ultimately though, success is determined by a measure of functional recovery.

Adult kidney stem cells have been used to repair renal tissue. One example is the proximal tubule stem cell identified by Gupta *et al.* (2006). It showed up to 10% integration into

renal epithelial tissue in a rodent ischemia-reperfusion injury model. However, there was no improvement in renal function as measured by serum creatinine or creatinine clearance.[34] Similarly, another study injected proximal tubule stem cells under the capsule after ischemia-reperfusion, and though it showed no variation of serum creatinine or blood urea nitrogen (BUN), it did result in a reduction of tubule impairment as indicated by urinary N-acetyl-beta-D-glucosaminidase (U-NAG) measurement.[35] Interestingly, this cell line spontaneously formed tubule-like structures when implanted into muscle. In a model of tubulonecrosis, CD133+ human tubule stem cells were injected intravenously into mice and showed localization and increased integration into site of injury, whereas this response was minimal in uninjured control animals.[76] Other kidney stem cells were tested in models of kidney repair. Papillary stem cells have been shown to integrate into renal medulla during transient ischemia.[81] Glomerular parietal cells selected for CD24+ mesenchymal stem cell marker were implanted in a clot under the capsule and showed signs of new glomerular and tubular tissue formation with recruitment of vasculature and expression of mature markers; however, cells only activated under compensatory response from contralateral nephrectomy.[74] Unfortunately, in all these adult kidney stem cell repair models, there was no functional improvement demonstrated. However, in one study, a reduction in proteinuria was achieved with repeated intravenous injections of Bowman's capsule stem cells into mice with Adriamycin-induced nephropathy. These cells could be expanded *in vitro* and differentiated into either podocytes or tubular cells. In addition, injected cells contributed to these populations *in vivo* after injury.[77]

Extrarenal adult stem cells have also been investigated for cell-based kidney repair. It was shown that an infusion of bone marrow cells reduced mortality, improved renal function, and reduced macrophage invasion in a rat glomerulosclerosis model. Higher doses of bone

marrow cells induced regeneration of mesangial and glomerular endothelial cells.[82] In 2007, it was reported that an intrarenal arterial injection of multipotent stromal cells (MSCs) reduced glomerular adhesion formation, proteinuria, and serum urea nitrogen in a rat model of glomerulonephritis.[83] However, in the long term (60+ days) there was histological evidence of sclerosis and adipocyte maldifferentiation. The most beneficial aspect of mesenchymal stromal cell therapy may actually be enhancement of their postulated role in renal repair: angiogenesis, reduction of inflammation, and stimulation of resident stem cell populations through paracrine signaling.[84] As of 2012, clinical trials for use of MSCs in ischemia-reperfusion injury are being conducted.[85]

An alternate source of cells for renal repair is amniotic fluid stem cells. Human amniotic fluid cells selected for CD117+ have been shown to increase tubular proliferation, reduce apoptosis, relieve tubular necrosis through cell protection, and recover function in immune deficient mice. These cells do not induce tumor formation, a particular concern for pluripotent cell lines.[32] Therapies based on amniotic fluid stem cells have potential to treat renal injuries, however, very few have shown functional recovery let alone long term efficacy. In addition, these studies have mainly shown repair through cell protective mechanisms with minor evidence of engraftment. Some renal diseases cause structural damage to the extracellular matrix, to which there is insofar no evidence of repair.

Tissue Structure Generation

Certain cell-based therapies have been shown to generate neo-kidney tissue when implanted into a kidney or ectopic site.[35, 80] However, strategies are also being pursued to generate renal structures using developmental tissues. Qiao *et al.* (1999) showed that ureteric bud tissue could be separated and induced to branch *in vitro* using only soluble media factors. Further, implantation of branched ureteric bud into a pool of metanephric mesenchyme induced

formation of nephron-like structures. It was suggested that developmental rudiments could be isolated, expanded, and combined to form complex kidney structures.[86] In a later study it was shown that such neo-kidney structures would recruit vasculature when implanted under the kidney capsule.[87] Recent studies demonstrated how autologous multipotent stromal cells could be placed in a xenoembryo at location of a ureteric bed before branch initialization and generate nephron-like structures. These structures could then be implanted back into donor omentum, recruit vasculature, and produce urine.[88] Such approaches are still at the proof of concept stage and require the use of xenoembryos, but isolation of the factors and developmental steps required to differentiate structures from autologous cells may lead to a completely *in vitro* procedure for generation of functional renal tissue.

Implantable Artificial Constructs

Renal dialysis is a time consuming process. To improve patient independence and compliance, there is sustained effort to produce miniaturized portable dialysis systems. Ronco *et al.* (2011) summarizes recent advancements. Notable device designs include the WAK (wearable artificial kidney) and ViWAK (Vicenza wearable artificial kidney). The former uses low flow continuous perfusion, while the latter employs a daily process of loading the peritoneal cavity with 2 liters of fresh solution followed by equilibrium, circulation, ultrafiltration, and fluid exchange. In order to lengthen the time between treatments, methods for glucose and bicarbonate regulation and small molecule removal are being investigated. Reduction in size and increased functionality and efficiency will correlate with improvements in nanoscale materials, micromechanics, and microfluidics.[89] Parallel efforts aim to use seeded renal cells on artificial constructs in order to reproduce the resorptive, homeostatic, metabolic, and endocrinologic functions of the kidney. One notable study from Lanza *et al.* (2002) used cells from a cloned bovine metanephros. These cells were seeded onto collagen I coated polycarbonate membranes

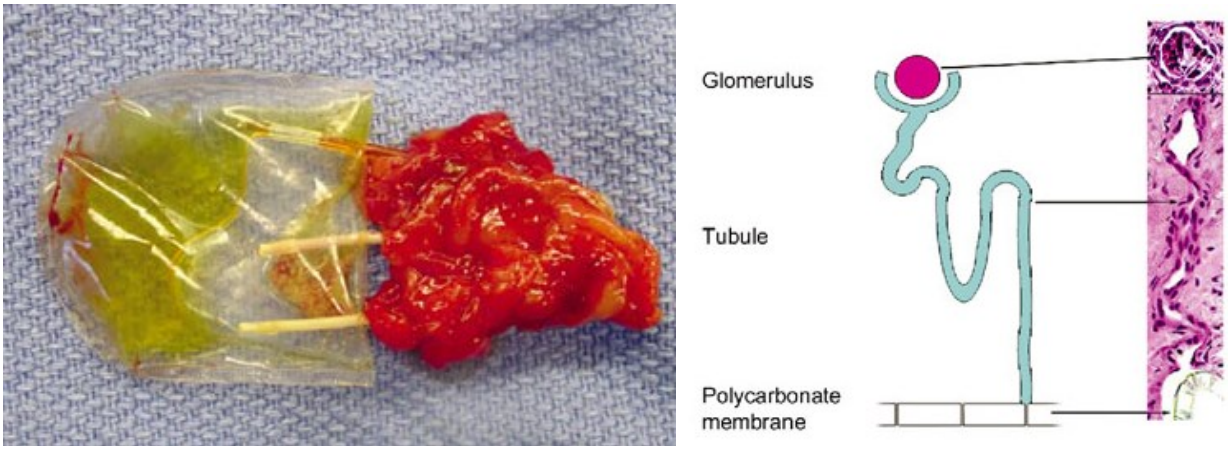

Figure 2-4. Tissue-engineered renal units. Cloned bovine kidney cells self-assemble around collagen coated polycarbonate tubes which drain into a polyethylene reservoir through silastic catheters. A). Constructs produce urine after three months of subcutaneous implantation. B) Histochemical analysis shows continuity between glomerulus, tubules, and polycarbonate membrane. Adapted by permission from Macmillan Publishers Ltd: Nature Biotechnology. Lanza RP *et al*. Generation of histocompatible tissues using nuclear transplantation. vol. 20, pg. 692, (Figures 3 & 4), copyright 2002.

and self-associated into renal units with connected glomerular and tubular structures (Figure 2-4). Implantable constructs assembled from these units recruited vasculature and produced urine-like fluid when implanted subcutaneously.[90] The Renal Assist Device (RAD) is another notable bioartificial device using renal cells. It pairs a conventional hollow-fiber dialyzer which acts as a hemofilter with a second dialyzer containing a confluent layer of renal tubule epithelial cells.[91] This improves the absorption and reclamation functions of the system, and has been shown to benefit patients with acute renal failure.[92] Currently, this type of system remains extracorporeal. However, in order to convert this type of system into a self-contained, persistent, implantable artificial kidney, design challenges and necessary technological improvements such as *in vivo* compartmentalization and membrane filtration efficiency, thickness, uniform porosity, biocompatibility and mechanical integrity are being addressed.[93, 94] Conditions have already been described for a renal epithelial cell bioreactor.[94] The culmination of these efforts to produce an implantable artificial kidney has resulted in the implantable Renal Assist Device (iRAD) project.

In 2012, in order to find an expedient solution for ESRD, the FDA announced that devices such as the iRAD and WAK would be fast tracked.[95]

Tissue Engineering Scaffolds

Biomaterials have been used to deliver cells to a site of intended kidney repair; support cell containment, attachment, and polarization; mediate destructive host responses; and even promote cell differentiation and structure assembly.[74, 80, 90] The overall goal is to provide a protected bioactive environment that will promote cell viability and eventual assembly into functional tissue structures. Biomaterials may be synthetic or tissue derived. A simple example of biomaterial use is the delivery of mouse CD24+ parietal epithelial cells into kidney parenchyma using clot gathered from an autologous tail vein bleed. The clot, a simple biomaterial, served to keep the cells localized.[74] In contrast, another study implanted an assortment of biomaterial constructs into a kidney *in vivo* to test histological response and determine the most suitable material for an implantable cell-material construct. Tested biomaterials included combinations of gelatin, hyaluronic acid, polycaprolactone (PCL), and poly(lactic-co-glycolic acid) (PLGA). Gelatin hydrogel was the most promising material to use in the construct according to measures of histopathology such as inflammation, fibrosis, necrosis, and mineralization, and measures of biocompatibility including degradation, neovascularization, and neotissue formation. The resulting primary renal cell/gelatin hydrogel implant promoted cell assembly into new renal structures.[80] In an alternate biomaterials application, a layer of collagen I was used to anchor developmental kidney epithelial cells to polycarbonate membranes in the construction of a small renal implant that produced urine-like fluid.[90] These examples of biomaterials used in renal tissue engineering are relatively simple in structure, however, given the architectural complexity of renal tissue, a tissue scaffold may be more effective in guiding kidney tissue formation.

A tissue scaffold has been described as optimally having four functions or features: architecture, mechanical stability, cell and tissue compatibility, and bioactivity.[96] Tissue appropriate architecture is the structure that has space and shape for functional cell arrangement, vascularization, and nutrient and metabolite transfer. Mechanically, the structure needs to withstand expected stresses at site of implantation while maintaining architectural stability. Additionally, as mechanical properties such as tissue stiffness can affect differentiation tendencies, it is desirable to match the scaffold and target tissue. Compatibility is the ability of the scaffold to integrate into recipient tissue and allow for cell attachment, growth, and differentiation without causing adverse tissue response. Bioactivity is the active regulation of cellular behavior. This may be an intrinsic feature of the scaffold or bestowed through modification. For example, ligands may be embedded in a scaffold to promote cell proliferation and differentiation.

Complex biomaterial scaffolds have been fabricated for use in tissue engineering. The goal was to mimic the normal extracellular environment of a tissue. Intricate porous structures have been created from polymers using gas-based techniques that include foaming, CO_2-water emulsion templating, and use of high-pressure CO_2 during crosslinking. They have been shown to allow for cellular infiltration, vascular invasion, and nutrient and waste movement.[6] Another current fabrication technique for artificial scaffold production is electrospinning. It uses electrical charge to drive a polymer solution into nanoscale fibers that may be layered to form a mesh. These meshes have been produced from synthetic polymers and ECM proteins such as laminin or PCL and collagen, and have also been embedded with bioactive peptides.[7-9] Despite these advances, artificial scaffolds cannot yet mimic the heterogeneous structures and elaborate chemical landscapes that are contained in natural ECM, and are therefore unlikely to reproduce

the *in vivo* microenvironment necessary for cell behavior appropriate to the tissue.[97] Fortunately, various methods exist to isolate intact extracellular matrices from tissues for use as cellular scaffolds.

Extracellular Matrix Scaffolds

Natural extracellular matrices, given their tissue specific structures, present as ready-made tissue scaffolds. They can be isolated from tissues using a cell removal process called decellularization.[11] This process generally diminishes bioactivity and structural integrity for the sake of biocompatibility. It has been shown, in regard to cell growth and directed stem cell differentiation, that culture with intact whole ECM is superior to heterogeneous mixtures of ECM proteins, which is in turn superior to individual matrix components.[20, 30] Implanted ECM has been shown to promote angiogenesis, recruitment of circulating progenitor cells, and constructive tissue remodeling.[49] Non donor-matched extracellular matrix scaffolds from both human and xenogeneic sources have been implanted clinically for the sake of tissue repair. Extracellular matrix constituents are generally conserved across species and it is possible to prepare xenografts that are immunologically tolerated that do not trigger cell-mediated implant rejection.[40, 97, 98] The ability to implant non-human ECM grafts significantly increases the pool of available scaffold material. Scaffolds derived from human and porcine tissues have been implanted clinically. These include bone, skin, bladder, nerve, heart valve, and small intestine.[12-17] Acellular matrices have also been produced from esophagus, ureter, tendon, ligament, skeletal muscle, artery, and even amniotic membrane.[99-107] A recent trend is the decellularization of organs for the intended purpose of whole organ regeneration; these scaffolds have been created from heart, lung, liver, and pancreas.[18-27]

Tissue Decellularization

The removal of cells from a tissue, or decellularization, leaves a residual extracellular matrix. This matrix can then be used as a scaffold for cellular repopulation. The process of decellularization can involve physical methods such as deformation, pressurization, agitation, sonication, or freeze-thaw cycles; chemical methods such as acid, base, alcohol, or detergent exposure, chelation treatments, or use of osmotic stress; or enzymatic methods to degrade cellular material or inhibit endogenous ECM-degrading proteases.[11, 108] Each step in a decellularization protocol can produce a unique result dependent on tissue, location, species, and age.[49] The overall goal is creation of a scaffold that evokes maximum cellular response with minimum immunogenicity.[109] This is achieved with removal of cellular debris, including DNA and other cellular antigens, and preservation of extracellular matrix proteins and structure.[108] The primary challenge is that each step in the cell removal process can have a harmful effect on native ECM, such as denaturation of cell adhesion peptides, so the procedure must be carefully balanced between cell removal and the destruction or removal of native ECM proteins.

The most prevalent method for tissue decellularization is treatment with biological detergents.[11, 108] Detergents disrupt lipid-lipid and lipid-protein interactions and are used to break apart cell membranes and carry away cellular material. Detergent properties are derived from their amphiphilic nature. The charge of the head group defines the detergent as non-ionic, cationic, anionic, or zwitterionic. The measure of detergency, or detergent effectiveness, is dependent on the critical micelle concentration (CMC). The CMC is the concentration at which a micelle will form. Below the CMC, detergent molecules will incorporate into a cell membrane, but detergent self-association including lipid-detergent and protein-detergent complexes will not form unless the CMC is exceeded. Detergents with low CMCs form more stable micelles with slow molecule incorporation. The CMC varies with temperature, pH, strength of counterions,

and presence of proteins, lipids, and other surfactants.[110] Ionic detergents can modify protein structure and disrupt protein aggregates to a greater extent than non-ionic or zwitterionic detergents. They are therefore more effective at cellular debris removal but may also cause more damage to basement membrane proteins. Factors such as head group, concentration, and duration of exposure all affect the outcome of a decellularization.[11] Detergent-based protocols have been particularly effective at decellularizing whole organs.[18-27, 108] The solutions are typically perfused through the vasculature, efficiently delivering to all areas of the organ.[28] The establishment of an effective detergent-based decellularization protocol for the production of kidney whole organ scaffolds would be a significant step toward the possibility of a functional tissue engineered kidney.

Cells for Kidney Tissue Engineering

Once a tissue engineering scaffold is produced, the greatest challenge is to repopulate it with the necessary cells in functional arrangement. There at least 26 identified cell types in an adult kidney.[58] Potential sources include adult kidney somatic cells, adult renal stem cells, extrarenal stem cells, pluripotent stem cells, and cells involved in kidney organogenesis. In practical application, the selection will depend on not only which cells will respond to the particular scaffold and growth environment, but which will be tolerated by the host upon implantation. In addition, methods for efficient isolation, purification, expansion, and differentiation are necessary to achieving sustainable and reproducible outcomes.

Adult kidney parenchymal cells, after injection into a kidney, have shown promise by self-assembling into kidney-like structures.[80] These structures have not been functionally tested, but it would be worthwhile to see if this behavior could be reproduced in a controlled *in vitro* environment with a kidney-specific scaffold. The primary drawback to this approach is that it would likely require destruction of a human kidney to harvest the cells. In addition, the cells

would need to be HLA-matched and the maintenance and balance of the many cell types over more than short term would likely not be feasible for routine application. The alternative approach is to use stem cells.

Adult Stem Cells

Stem cells have a high capacity for proliferation and the potential to differentiate into multiple mature cell types. The derivation of maintenance culture conditions and establishment of stem cell lines may provide an abundant cell source for organ regeneration. The challenge is to identify the stem cell or limited combination of stem cells which have the potential to differentiate into all cell types necessary for kidney function. A review of promising stem cell sources is provided by Harari-Steinberg *et al.* (2011).[39] Several adult renal stem cell candidates have been identified, typically through expression of mature and embryonic markers, reduced or prolific cell division, activation during injury repair, and ability to integrate into a developing kidney.[34-37] Locations of proposed stem cells have included proximal, distal and collecting tubules, interstitium, glomerular parietal epithelium, and papillae.[34-38, 77] These multipotent cells have been shown to differentiate into adult kidney cells and contribute to renal tissue after injection into developing metanephros.[34, 36, 37] In addition, they have been induced into non-renal lineages.[34, 37] Extrarenal stem cell sources such as hematopoietic stem cells and MSCs are another option for scaffold repopulation. Examination of current injury repair models reveals they do not normally contribute to nephrogenic cell lineages.[38, 39] However, similar to adult kidney stem cells, they have been induced to differentiate into various alternate tissue cell types.[111] In addition, it was shown that human MSCs will differentiate and contribute to glomerular epithelial, tubular epithelial, and interstitial cells if implanted in a cultured embryonic rodent kidney.[112] Unfortunately, culture conditions to achieve this *in vitro* have not been defined. Unless these adult stem cells can be implanted without eliciting a rejection response, they will

have to be extracted from the host or donor matched. Hematopoietic stem cells and mesenchymal stromal cells have the advantage of being isolated from peripheral blood.[111] The attraction of adult stem cell use for kidney tissue engineering is the opportunity for autologous sourcing, yet there is no assuring that any combination of these cells can account for the totality of adult renal somatic cells.

Fetal Stem Cells

All cells in the kidney are derived from the intermediate mesoderm. From there branching networks of transitional cell populations progress, interacting and differentiating until all cells in a mature kidney are accounted for (Figure 2-3). These precursor cell populations, able to differentiate into multiple mature renal cell types, may be candidates for use in scaffold-based tissue engineering. One cell line, 7.1.1, has been derived from rodent metanephric mesenchyme and will differentiate into all renal cells except for those originating from the ureteric bud epithelium.[81] Other possibilities include the Six2+ Cited1+ stem cells from the cap mesenchyme, from which all the epithelial cells in the nephron originate, or the Foxd1+ stem cells, precursors to the stromal population.[38, 113] These potential stem cell sources still need defined culture conditions that would allow *in vitro* maintenance with preservation of stemness.

Cells from dissociated metanephros have been shown to self-assemble into glomerular- and tubular-like structures.[90] Further, it has been shown that cells from a fetal organ will infiltrate an acellular scaffold when co-cultured adjacently.[114] An advantage of using cells from a fetal kidney is their inherent propensity to differentiate into mature kidney cells.[39] In an effort to create a histocompatible source for these embryonic kidney cells, somatic cell nuclear transfer (SCNT) was utilized (Lanza, 2002). This technique, also considered therapeutic cloning, involves insertion of the nucleus of a somatic cell from the host into a donor egg, initiation of

cell division, and implantation of the resulting embryo into a surrogate. The developing organ of interest may then be used at the target time point.

Realistically, it is unlikely that human fetal cell sources or SCNT methods will be employed. First, the manipulation of human fetuses for the purposes of cell harvest has strong ethical considerations. Second, SCNT has been largely superseded by the creation of induced pluripotent stem cells (IPSCs), autologous cells modified to have similar properties to embryonic stem cells.[33]

Pluripotent Stem Cells

Pluripotent stem cells have the ability to differentiate into any cell derived from the three germ layers, endoderm, mesoderm, and ectoderm. This makes them attractive for scaffold-based tissue engineering as one cell source may provide all necessary cell types for organ function.[29] Given this plasticity, the primary challenge is to direct cells to intended lineage without maldifferentiation or teratoma formation.[39] Pluripotent cells include embryonic stem cells, amniotic fluid stem cells, and induced pluripotent stem cells. They each can form embryoid bodies, three-dimensional aggregates capable of *in vitro* tissue formation.[31-33] Also, each have been directed toward renal lineages using prescribed cell culture conditions.[32, 115] Another advantage to pluripotent stem cells is they likely have a proliferative capacity that exceeds adult or fetal stem cells.[39]

Embryonic stem cells. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst stage embryos.[116, 117] They are easy to maintain, have a high doubling capacity, and can be passaged indefinitely.[31, 116-118] There are established cell lines from human and mouse, among others, and there is an extensive body of knowledge in handling, differentiation, and application.[31] In regard to renal differentiation, teratomas developed from implanted ES cells were shown to spontaneously form branching ducts with nephrogenic

morphology and express kidney-specific markers.[119] Also, ES cells in culture have been directed to differentiate toward mesoderm, intermediate mesoderm, metanephric mesenchyme, and more mature tubule cell types through addition of growth factors and other additives to the medium; these include BMP2 (bone morphogenetic protein), BMP4, BMP7, GDNF, HGF (hepatocyte growth factor), Activin, and retinoic acid.[120-123] Establishment of human ES cell lines requires manipulation of embryos, which gives rise to ethical implications. Initially, establishment of ES cell lines required embryo destruction, yet it has also been accomplished using a non-destructive blastomere biopsy. This technique removes a cell at the 6-8 cell stage of an embryo and is used by fertility clinics for pre-implantation genetic diagnosis and selection.[124] Unfortunately, differences between biopsied and unaltered embryos have been identified and long term effects of the surviving embryos are uncertain.[125, 126] Currently, at least two-hundred human ES cell lines are available for use.[127]

Amniotic fluid stem cells. In order to test for chromosomal abnormalities or infection during pregnancy, fluid may be withdrawn from the amniotic sac in a procedure called amniocentesis.[128] Roughly one percent of the cells in the extracted fluid have been identified as stem cells with either c-Kit (CD117) or Oct4 positivity.[32, 129] These amniotic fluid stem (AFS) cells are highly proliferative, have been differentiated into cells from all three germ layers, and if positive for both Oct4 and c-Kit form embryoid bodies. Amniotic fluid stem cells have cell surface markers and gene expression patterns that are distinct from ES cells.[32] In addition, they do not develop spontaneous tumors when implanted into immunodeficient mice.[129] There is no current AFS cell therapy, but these cells have been shown to integrate into adult tubules and early nephron structures.[32, 130] Also, using growth factors, AFS cells have been differentiated into mesenchymal and epithelial cells using growth factors, with one derivation showing expression

of a podocyte marker.[32] Despite these achievements, there is still little research in directed differentiation of AFS cells into diverse renal lineages. Finally, one specific drawback of routine use of amniotic fluid stem cells is that there is a minor risk of miscarriage associated with amniocentesis.[128]

Induced pluripotent stem cells. Embryonic stem cells are an attractive source for tissue engineering applications because of pluripotency and high proliferative capacity. However, potential immune response and ethical considerations in obtaining these cells are substantial drawbacks. One solution is to reprogram adult autologous cells into a pluripotent stem cell state. This has been accomplished with human dermal fibroblasts by retroviral transduction of four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc. The resulting cells, dubbed induced pluripotent stem cells, mimic embryonic stem cells with similar morphology, proliferative capacity, gene expression, surface antigens, and telomerase activity.[33] They can be differentiated into mesodermal, endodermal, and ectodermal lineages through embryoid body formation, and have been differentiated to cells such as neurons, cardiomyocytes, adipocytes, and pancreatic β-islet cells.[122, 131, 132] The primary foci of IPSC research has since been identification of new accessible cell sources and devising reprogramming protocols for minimal modification and removal of the genetic imprint.[133] Once the cells reach the embryonic state, lingering modified expression patterns can inhibit or influence differentiation potential.[122] Recent efforts of nuclear reprogramming include factor substitution or process efficiency improvement with use of small molecules, direct delivery of reprogramming factors, and transfection of synthesized mRNA.[133] Induced pluripotent stem cells have also been generated from bone marrow cells, kidney mesangial cells, cord blood stem cells, mesenchymal cells in cord matrix and placental amniotic membrane, peripheral blood cells and exfoliated tubule cells excreted in urine.[43, 44, 134-136] The

primary advantage to IPSC as a cell source is that reprogrammed autologous cells can be used to generate tissue without immunogenic rejection.[137] Further, cell sources can now be easily acquired from urine or peripheral blood without need for tissue biopsy.[44, 138] One caution is that adult cells may be susceptible to age, mutation, and disease.[135] As an alternative, cord blood, umbilical cord matrix, and placental amniotic membrane can be banked with little ethical concern for donor matched applications.[134, 135]

Embryonic stem cells and induced pluripotent stem cells have been induced to differentiate to similar renal lineages. In one study, both achieved mesoderm, metanephric mesenchyme, and tubule differentiation. However, ES cells were more responsive to differentiation factors and IPSCs had delayed loss of Oct3/4 expression. This may be related to the method of cell reprogramming and continued expression of transduced transcription factors.[122] Relatedly, it has also been shown that IPSCs have a predisposition to differentiate toward their source tissue type due to epigenetic patterning (histone modification or DNA methylation), and are more resistant than native ES cells to progress to intended lineages.[133] This predisposition may be partially mitigated by progressing the cells to intended phenotype and then reverting; however, this may not be practical in application.[139] Finally, as with ES cells, given high proliferation and diverse differentiation potential, use of these cells carries a risk of teratoma formation.

CHAPTER 3
KIDNEY DECELLULARIZATION

Experimental Plan

Introduction

The production of tissue engineering scaffolds through the decellularization of tissue has been repeatedly shown to be successful with an assortment of tissue types. These scaffolds provide a biologically active tissue-appropriate template for cellular repopulation with the ultimate goal of regenerating a functioning organ. A whole organ preparation is especially valuable as it retains organ-shaped three-dimensional extracellular histoarchitecture. It has been postulated that only an intact organ extracellular matrix best represents the conjoined tapestry of microenvironments *in vivo* and is therefore best able to facilitate the generation of whole functioning complex tissues.[140] This is particularly relevant to the kidney, as its vital filtration function requires a precisely coordinated arrangement of numerous cell types with heterogeneous underlying extracellular matrices.

There are many avenues for decellularizing tissue, including a combination of mechanical agitation or strain, freeze-thawing, osmotic disruption, enzyme exposure, and acid, base and detergent wash.[11] The selection of the optimal protocol is dependent on the type of tissue, and factors to consider are cellularity, density, lipid content, and size.[108] In the past several years, decellularization protocols have been successful in producing intact whole organ extracellular matrix scaffolds. The primary mechanism for cell removal is perfusion of detergent-based solutions through the innate vascular network via cannulation of the arterial supply. This approach for fluid delivery takes advantage of the intrinsic system for oxygen and nutrient distribution and not only maximizes exposure of the tissue to the decellularization solutions but provides an avenue for resulting cellular debris removal. Typically, once the organ is

decellularized, the vascular network remains intact and may then be used as convenient system for cell delivery and distribution. Whole organ scaffolds have been successfully produced from lung, heart, and liver using perfusion-based decellularization. They each showed preservation of key extracellular matrix proteins and effective removal of DNA. Further, each has been used as a scaffold for organ-specific cellular repopulation.[18, 20-27]

A protocol for rabbit whole organ kidney decellularization was published in 1979 by Brendel *et al*. Detergents and enzymes were circulated into the renal artery in a specific sequence over two days to produce a translucent achromatized kidney.[141] However, the purpose of the protocol was for the isolation and study of the renal extracellular matrix; specifically the glomerular basement membrane (GBM). The study showed that the produced renal ECM had a preserved basement membrane ultrastructure and retained a degree of glomerular charge and size selectivity. Perfusion of a red blood cell solution through the preserved vascular basement membrane network showed no leakage of cells across the GBM and into the tubules; in addition a collectible urinary filtrate was produced. Given the intention of the protocol and that it was developed before the advent of tissue engineering and regenerative medicine, it was never assessed as a potential cellular scaffold. In the following year, that same group released a paper that contained a simpler vascular basement membrane (VBM) preparation protocol.[142] That protocol was designed to be used on multiple tissues, including rat kidney, and it is that protocol which was first evaluated in this research for production of a whole kidney tissue engineering scaffold.

The remainder of this chapter will detail initial experimental plans and results, protocol improvement strategies and subsequent results, a discussion of the approaches, and considerations for future work in regard to the production an acellular kidney scaffold with

potential to support renal tissue engineering applications and the eventual generation or repair of functional renal tissue. Detailed methods and materials are described in Chapter 6.

Organ Harvest

Kidneys for use in the decellularization experiments were harvested from Sprague-Dawley rats using a variation of the protocol described in Adin *et al.* (2005). This procedure allowed for the isolation and direct cannulation of the right renal artery *in situ*.[143] In addition, administration of an anticoagulant and vasodilator minimized clotting and vasoconstriction during kidney harvest. Once the cannula was secure, the organ was rinsed with physiologic saline, extracted, and immediately attached to the decellularization system. The organ was suspended by the cannulated renal artery and connected to the perfusion system via a Luer fitting. Optionally, the ureter may have been cannulated before kidney removal to measure urinary filtrate. During the procedure, the animal was under full anesthesia and once the organ was extracted it was euthanized. All animal work was done humanely and according to guidelines supplied by the Institute for Lab Animal Research Guide for the Care and Use of Laboratory Animals and approved by the University of Florida (UF) Institutional Animal Care and Use Committee.

Kidney Decellularization

Decellularization of the kidney was performed with the LifeSustainer 1000 automated organ perfusion workstation. It was introduced in Sadri *et al.* (1997) and used for organ perfusion experiments in Adin *et al.* (2005).[143] This system perfuses a small animal organ with constant flow rate or pressure at a desired temperature.[144] In addition, sensors for pH, oxygenation, and carbon dioxide saturation allow this system to be used for any subsequent perfused organ culture experiments. In the following experiments, harvested kidneys were perfused in a pulsatile manner within rat physiologic tolerances (~120/80 mmHg). The perfusate

was drawn from a reservoir and once passed through the kidney either recirculated through a filter or collected for disposal or analysis.

The decellularization protocol described by Brendel and Meezan (1980) is a stepwise perfused-organ protocol that was designed to lyse cells and progressively remove material without causing blockage; this is especially relevant in the miniscule tubular network of the kidney.[142] The protocol, *VBM*, is detailed in Chapter 6 (Table 6-1). Briefly, 2% Triton X-100 was perfused through the organ for 48 hours to solubilize hydrophobic cell membranes, lyse cells, and wash cytoplasmic contents; followed by buffered DNase to digest any bulky, viscous nuclear material; and then more Triton X-100 to assist in further cellular debris removal. Interspersed were de-ionized water flushes to osmotically challenge remaining cells and rinse solutions.

Scaffold Analysis

Initial evaluation of the decellularized kidneys was accomplished with routine histology. The prepared scaffolds were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and viewed using bright field microscopy. This preparation method has a high degree of tissue structure preservation and hematoxylin highlighted any DNA remaining in the scaffold. DNA and other cell debris can hamper cell growth and trigger destructive immune response in an implanted extracellular matrix scaffold.[40, 145, 146] The sections of tissue were interpreted for removal of cell debris and retention of extracellular morphology. Specifically, the functional regions or zones of the kidney, the cortex and the outer and inner medulla, were inspected for intact cell or nuclear membranes, DNA remnants, and other cellular debris. In addition, the vascular, glomerular, and tubular basement membranes were inspected for expected morphology and continuity. Further analyses such as electron microscopy and protein IHC supplemented successful outcomes.

Results

Scaffold Production

Kidneys from the SD rats were successfully cannulated, secured, and rinsed (Figure 3-1). The goal of the surgery was a complete, low-resistance organ flush with no visible areas of blood pooling; it was thought that such would be most amenable to the proceeding organ perfusion steps. Factors that contributed to success were speed of the surgery, control of hemorrhage, minimal organ manipulation, and securing the cannula without leakage.

Once the harvested and rinsed kidneys were secured to the perfusion system (Figure 3-1), the *VBM* decellularization protocol was initiated. However, it became readily apparent that the pressure control algorithms were having difficulty adjusting to the changes in both resistance and fluid viscosity experienced during a decellularization sequence. The result was periodic spikes in pressure as reported by the inline transducer. A switch to flow control showed some, yet not significant improvement. In addition, over time, miniature air bubbles appeared within the tubing and were not effectively removed in the air trap. In cases, it appeared these air bubbles worked into the perfusing kidneys and likely embolized many of the internal vessels. Efforts were made to control any air intrusion, but to no avail; the tubing fixtures and fluid switches in the system were likely ill-equipped to adapt to long term detergent perfusion. Regardless, the protocols were completed and decellularized kidneys were produced.

Scaffold Evaluation

Decellularized kidneys appeared white and semi-transparent and of a similar size and shape to the harvested kidneys (Figure 3-1). The organs were compressed and exhibited sponge-like re-expansion. Histological evaluation of the organs showed recognizable structures, but with very large cavernous defects and a great deal of proteinaceous debris. The cause of the defects was likely pressure spikes resulting from a combination of air emboli in the kidney vasculature

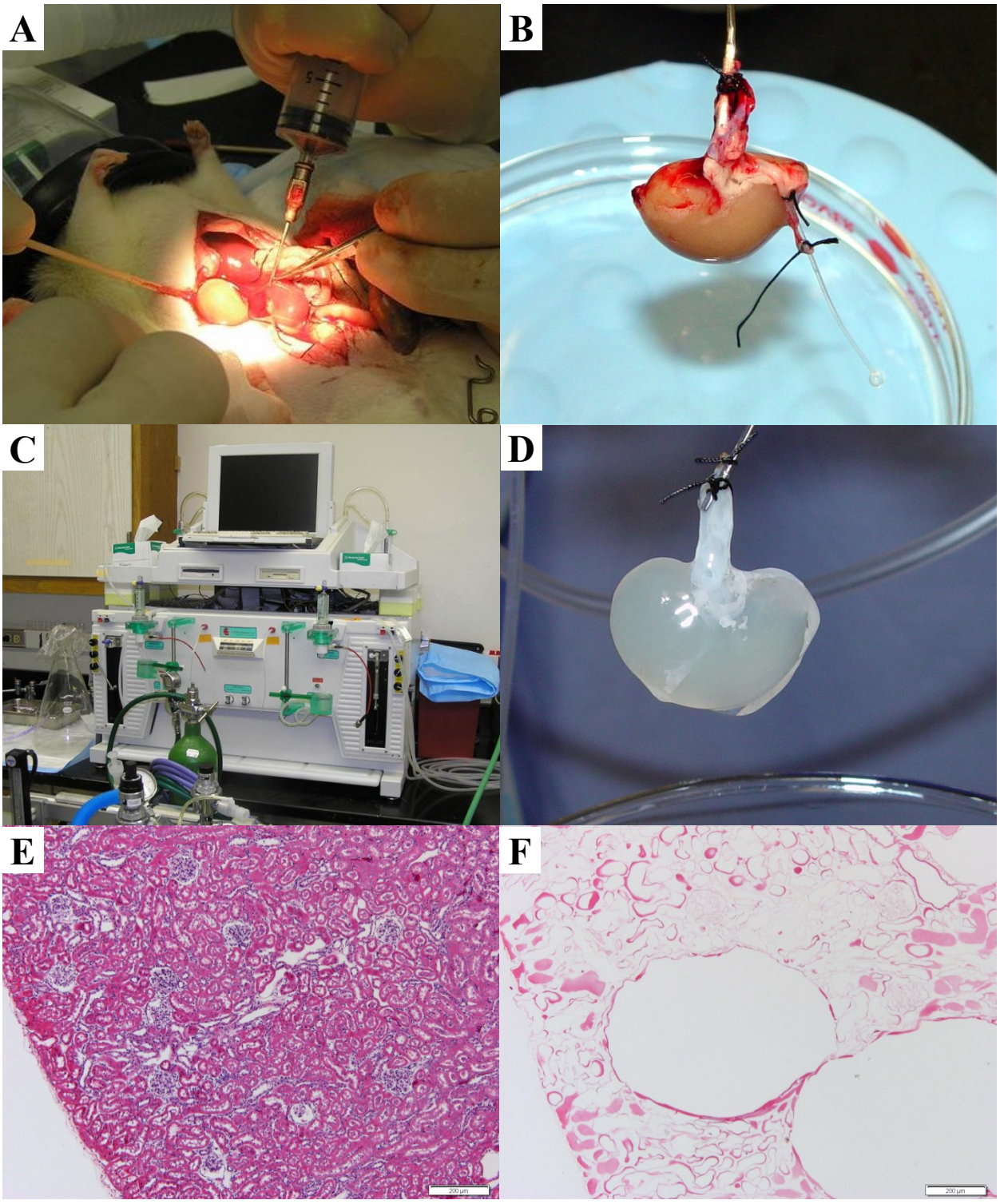

Figure 3-1. Organ harvest and perfusion-based decellularization. A) Kidney harvest with in situ saline perfusion, B) harvested kidney with both arterial and ureteral cannulation, C) LifeSystem 1000 organ perfusion system, D) decellularized kidney, and H&E stain (10x magnification, bars denote 200µm) of E) normal kidney and F) organ decellularized with machine-driven organ perfusion system; note the damage to ECM (*VBM Mod 1* protocol).

and rapid release of cellular lysates. In order to slow the initial release of cellular material during decellularization, a modified protocol, *VBM Mod 1*, was devised which decreased the detergent concentration of the first perfusate solution. This change was inspired by an earlier paper from Brendel *et al.* (1979) that targeted the preparation of an acellular rabbit kidney.[141] This change resulted in marginal improvement but the considerable damage to the ECM remained (Figure 3-1). Interestingly, despite this damage, it appeared most of the cells were lysed with only a few isolated patches of intact cellular or nuclear membranes. Also there were no identifiable areas of hematoxylin positivity, suggesting all of the DNA had been removed or was diffused within the visually eosinophilic proteinaceous debris.

Procedure Improvement

Elevated Reservoir Perfusion Apparatus

The initial results have shown that a structurally intact decellularized kidney could not be achieved with the LifeSustainer 1000 automated organ perfusion workstation. A system redesign to include retrofitted tubing fixtures, a mechanism to relieve pressure spikes, and modification of the control algorithms for improved pressure control would have been prohibitive in time and cost, especially given the system and control algorithms were proprietary and would require substantial reverse engineering. Therefore, a new, simpler apparatus was designed to perfuse kidneys.

The driving principle behind the new organ perfusion system was pressure control based on height of an elevated perfusate reservoir. Tubing from the reservoir directed the decellularization solutions through a vented inline filter, and then down through a split that allowed two organs to be attached. Perfusate that passed through the kidney was collected in a common beaker and then returned by peristaltic pump to the elevated reservoir. A transducer was placed at the level of the kidneys to assure desired pressure. Even though the principle is based

on a static fluid assumption, the pressure remained stable and was maintained as long as there were no leaks in the system or gross ruptures in the kidney. Design and images of the apparatus are detailed in the appendices (Figures A-1 and Figure A-2). This simple system filtered debris, minimized air entrapment, and provided a common solution source for two decellularizing organs. This apparatus was used for all subsequent scaffold preparations.

Decellularization Protocol Improvement

The Brendel and Meezan VBM decellularization protocol (1980), implemented with the LifeSustainer 1000 automated organ perfusion workstation produced kidney scaffolds with large extracellular matrix defects and substantial proteinaceous debris, yet successfully lysed most of the cells. To address the gross ECM defects, a new organ perfusion system with better pressure control was devised and constructed. However, further modifications to the decellularization protocol were also thought necessary to reduce residual cellular debris. Therefore, iterative adjustments were made such as increased detergent concentration and duration, the addition of ionic detergent and detergent additives, and the substitution of detergents. These changes and results are described below and summarized in Table 3-1. The protocols are fully detailed in Chapter 6 (Materials and Methods). Table 3-1 also includes the number of kidneys that were decellularized by each of these protocols, many of which were involved in cell implantation experiments described in the following chapter. It should be noted that the overall goal of this research was to produce an acellular kidney scaffold that could be evaluated as a cellular scaffold for tissue engineering, and ultimately success would be judged by cellular response. It was not the primary goal to necessarily optimize a protocol for detergent times, concentrations, and precise sequence. However, modifications were tested in order to identify potential parameters or arrive at a protocol which would result in a dramatic improvement in debris removal, as such a produced kidney was thought to be a superior baseline construct from which to evaluate.

Table 3-1. Decellularization quality of kidney scaffolds produced with the elevated reservoir perfusion apparatus and detergent-based protocols

Decellularization Protocol	#	DQ	Modification
Kidney Mod 1	2	G	Modification of Brendel and Meezan's Acellular Perfused Kidney protocol with addition of ionic NaDC and additional DNase step
Kidney Mod 2	3	G	*Kidney Mod 1* with lengthened detergent perfusion durations
Graduated 3%	2	G	Gradually increasing detergent concentrations
Graduated 10%	5	VG	Similar to *Graduated 3%* with higher concentrations
Kidney Extended	4	VG	Similar to *Kidney Mod 2* with longer detergent perfusion durations
Kidney NaDC	16	G	Similar to *Kidney Extended* with reduced detergent durations and additional detergent step
Kidney SDS	19	E	Equivalent to *Kidney NaDC* with substitution of SDS for NaDC

#: Number of scaffolds produced with given protocol; DQ: typical decellularization quality; G: good, glomeruli free of debris, patchy zones of debris in cortex, most debris located in outer medulla, diffuse debris patterns in inner medulla, possible isolated areas of intact cellular or nuclear membranes; VG: very good, all areas of kidney improved, minimal debris in cortex and inner medulla, some debris still in outer medulla, no intact cell remnants; E: excellent, only trace debris in entire kidney; NaDC, sodium deoxycholate detergent; SDS, sodium dodecyl sulfate detergent.

Table 3-1 describes a basic system for categorization of decellularization quality. Determinants include presence of intact nuclear envelopes and amount of residual cell debris in three areas of the kidney: cortex, inner medulla, and outer medulla. Pictorial examples of each category are represented in Figure 3-2. Note that these categories are based on an objective classification of the group as a whole, and do not assess the level of variation within the scaffolds produced by a specific protocol.

Acellular perfused kidney protocol. *Kidney Mod 1* was the first decellularization protocol that was compiled and used on the new elevated reservoir perfusion apparatus. It incorporated many of the steps that were used in Brendel and Meezan's acellular perfused kidney protocol (1979).[141] That protocol was optimized for rabbit kidney and preceded their subsequently published VBM protocol; their VBM protocol was the one used in these initial

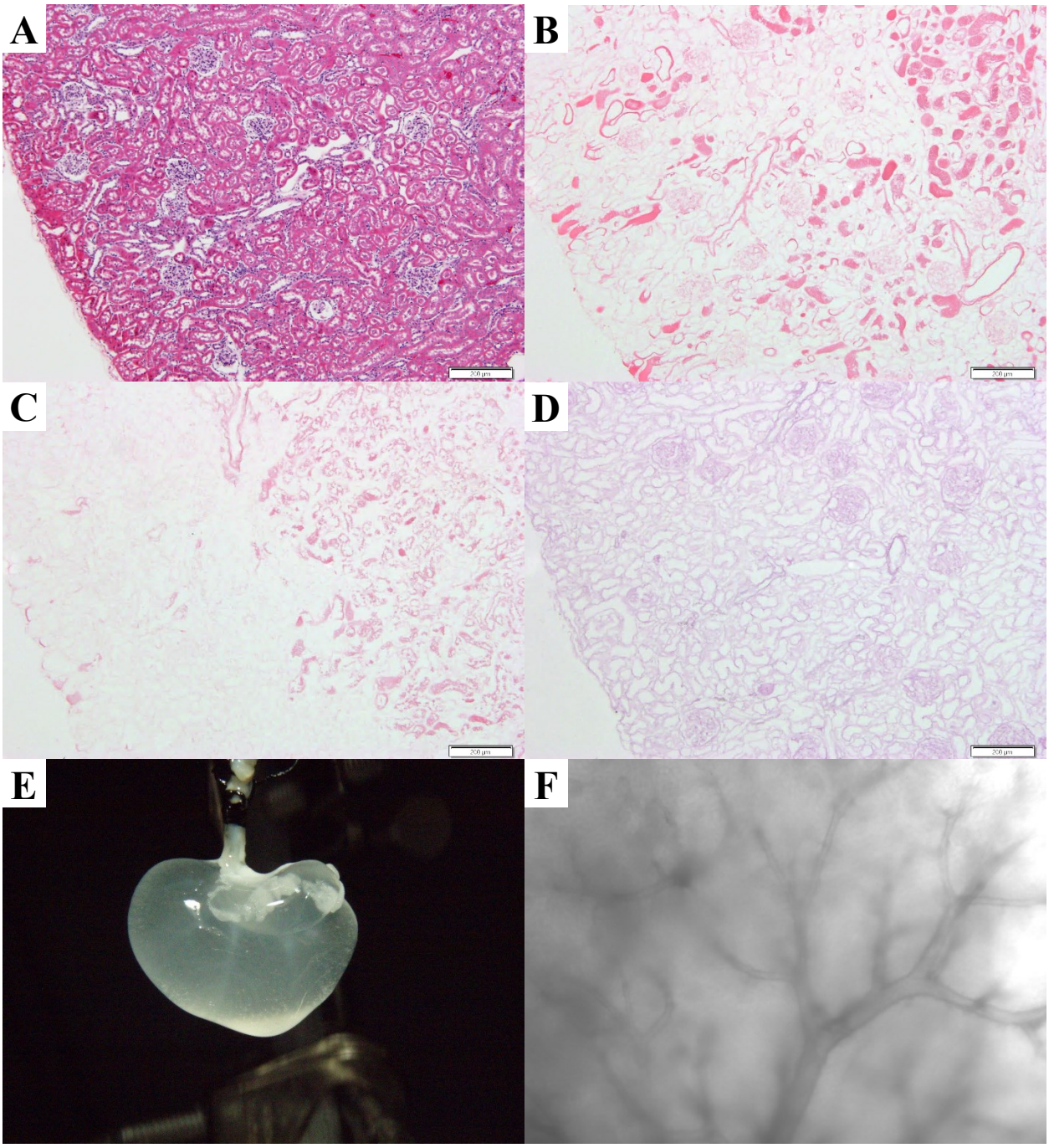

Figure 3-2. Improved decellularization protocols for removal of cell debris. A-D) H&E histochemistry (10x magnification, bars denote 200µm). A) Normal kidney. B-D) Protocols showing increasing decellularization quality, B) *Kidney Mod 1*: good decellularization quality, C) *Graduated 10%*: very good decellularization quality with reduced debris in the scaffold, D) *Kidney SDS*: excellent quality with only trace debris in the scaffold. E) *Kidney SDS*: image of whole decellularized kidney, note the increased translucency. F) *Kidney SDS*: Bright field image showing intact vascular network (4x magnification).

decellularization experiments (Brendel, 1980).[142] Even though the VBM protocol was considered an updated version, the acellular perfused kidney protocol included steps that appeared to be more aggressive and had the potential to be more effective at cell debris removal and removal of cellular membranes. In comparison to the previous VBM protocol, this protocol includes the detergent additives $CaCL_2$ and $MgSO_4$ to promote cell permeability and encourage activation of endogenous nucleases, NaCl to osmotically assist in nuclear membrane rupture, and the ionic detergent sodium deoxycholate (NaDC) to solubilize previously insoluble protein aggregates and improve cell debris removal. In addition, an intermediate DNase treatment was added to dissuade accumulation of cumbersome nuclear material, which even though not apparent at the conclusion of the previous *VBM* protocol (via H&E stain), may have still aggregated and blocked protein removal during the protocol. In an act of caution in contrast to these more aggressive steps, the durations of solution exposure was kept at a minimum.

Kidneys produced with the *Kidney Mod 1* protocol and with the elevated reservoir perfusion apparatus contained no ruptures of the extracellular matrix, and glomerular, tubular, and vascular basement membranes appeared intact and with expected morphology (Figure 3-2). All of the cells and a majority of eosinophilic cellular debris was removed from the cortex. However, cell debris was spread throughout the outer and inner medulla, with areas of intact nuclear envelopes. Lack of hematoxylin contrast suggested that the DNA had been successfully removed or perhaps that the remaining nucleic elements were diffused in the proteinaceous debris so as not to be apparent.

The next iteration of protocol adjustment, *Kidney Mod 2*, involved the lengthening of most of the detergent steps in *Kidney Mod 1* so that the total protocol duration was almost doubled, similar to the *VBM* protocol. Oddly, this did not yield notable improvement.

High detergent concentration. Two kidney decellularization protocols were developed which involved gradually increasing nonionic detergent (Triton X-100) concentrations, one with a peak concentration of 3% and one with 10%. This was done in an effort to temper any sudden release of cell lysates so as to not overwhelm the ability of the circulating perfusate to effectively wash away cellular material. The inclusion of DNase, detergent additives, and sodium deoxycholate steps were incorporated as in previous protocols, although these two protocols only contain one DNase step. The previous protocols only included a maximum 3% nonionic detergent concentration; the *Graduated 10%* protocol magnifies this significantly to explore the effects of higher detergent concentrations. Additionally, the *Graduated 10%* protocol included more steps with the addition of sodium chloride to improve lysing of nuclear membranes.

Histological analysis of acellular scaffolds produced with the *Graduated 3%* protocol showed no meaningful improvement over previous protocols. However, the *Graduated 10%* protocol yielded a scaffold with significantly reduced cellular debris and an absence of identifiable cellular remnants (Figure 3-2)

Extended duration. It was determined that a graduated increase of detergent level to a high concentration produced acellular kidneys with less cellular debris and a sharp reduction in identifiable cell remnants such as nuclear membranes. The next set of protocols tested the effects of exaggerated detergent exposure without the increase in concentration. Total protocol duration was extended to 7 days, with almost 6 days of Triton X-100 treatment with calcium chloride ($CaCL_2$), magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl). In addition, the NaDC duration was increased to 20 hours, at least double that of the previous procedures. The two protocols, Kidney Extended and Kidney Extended 2 only contained one difference; Kidney Extended had a DNase treatment interrupting the mentioned Triton X-100 step, whereas Kidney

Extended 2 did not. Fortunately, the acellular kidneys produced using either protocol showed a very good decellularization, with characteristics similar to the *Graduated 10%* protocol. In addition, there was little noticeable difference between the two protocols, calling into question the necessity of the intermediate and costly DNase step.

Convenient timing. Tested protocols have suggested that high detergent concentration and prolonged exposure positively affect cellular debris removal during kidney decellularization. The next protocol was developed to combine the more aggressive detergent steps of the acellular perfused kidney protocol (Brendel, 1979) with the convenient 24 hour based timing of the VBM protocol (Brendel, 1980).[141, 142] The resulting protocol is shorter in duration than the *Kidney Extended* protocols, yet retains the long sodium deoxycholate exposure. This was done to see if ionic detergent exposure was a strong factor for effective debris removal. Other changes were an extension of the DNase step and removal of the initial 0.5% Triton X-100 treatment.

The kidney scaffolds generated from the *Kidney NaDC* protocol unfortunately were not as good as the ones generated from the high concentration and extended duration protocols. However, at this time, scaffold cell-seed experiments were progressing and showed promising results on this preparation. Therefore, many of these scaffolds were produced to provide an unwavering baseline for the cell-seed experiments. The alterations in the decellularization protocol was not completely frozen, however, as a shift in ionic detergent proved to be a very successful as described in the next protocol.

Ionic detergent change. The two published procedures which inspired the decellularization protocols used in this research (Brendel, 1979; Brendel, 1980) were pursued because they had been designed for and tested on the kidney.[141, 142] However, the procedures were not developed for the purpose of production of a scaffold for tissue engineering nor

optimized for debris removal. A survey of contemporary published protocols for decellularizing tissue suggested a change in detergents may have yielded more promising results. Sodium dodecyl sulfate (SDS) has been used alone or in conjunction with other detergents or agents to decellularize tissue. It is an ionic detergent and in comparison to NaDC, SDS is reported to be superior in removal of nuclear remnants and cytoplasmic proteins. However, it has also been described as disruptive to native tissue structure and collagen integrity and results in a reduction of glycosaminoglycan (GAG) concentration; though on the whole less than NaDC.[11] Sodium dodecyl sulfate has been used to decellularize artery and vein, lung, liver, and heart.[20-23, 25, 26, 101, 103, 147] In order to evaluate SDS, a simple replacement of the ionic detergent NaDC in the *Kidney NaDC* protocol with an equivalent concentration of SDS was dictated (*Kidney SDS*). As all other parameters would remain fixed, a direct comparison could be made.

The decellularization protocol utilizing the SDS ionic detergent was a substantial improvement and produced kidneys with no identifiable intact cells and only trace eosinophilic debris. The renal extracellular matrices all appeared intact and with expected morphologies. The generated scaffolds as a whole appeared highly translucent, with easily recognizable vascular networks (Figure 3-2). Consequently, this protocol was used for the generation of numerous acellular kidneys for use in scaffold seeding experiments. Further, the response of cells to scaffolds produced using two different ionic detergents could be directly evaluated.

Finally, given the success of the use of SDS in decellularizing kidneys, and that SDS is effective at DNA removal, a simple preliminary experiment was conceived which subjected two rat kidneys to a one day perfusion of SDS followed by a deionized water flush (1-Day SDS).[26] The resulting scaffolds achieved debris removal similar to the full *Kidney SDS* protocol, indicating significant potential for time and step reduction.

Supplementary Scaffold Analysis

Scanning Electronic Microscopy

The primary criteria for determination of success of a produced decellularized scaffold in regard to the discussed series of protocols has been extracellular matrix morphology preservation and removal of cell lysates as interpreted by H&E histochemistry. However, other key factors for consideration were preservation of continuous three-dimensional structure and retention of essential extracellular matrix proteins. Scanning electron microscopy (SEM) is an imaging modality that provides a surface scan of a material at submicron resolution. It has been used on whole organ ECM scaffolds to make a general assessment of three dimensional topographical patterning, including basement membrane continuity, and to verify removal of cellular debris.[24, 27, 141, 148] Therefore, an SEM image was taken of a transverse kidney section produced with a high detergent exposure protocol (*Graduated 10%*). Despite this aggressive treatment, the scaffold showed remarkable preservation of the visualized extracellular matrix, and only sparse areas of protein debris (Figure 3-3).

ECM Immunohistochemistry

One concern with decellularization treatments, especially those with high detergent concentrations, excessive detergent exposures, and ionic detergent use is damage to the extracellular matrix proteins that are necessary for constructive cellular response.[11, 20, 22, 108, 149] Immunohistochemistry is a histological technique that employs antibodies for contrasting target proteins on a tissue slide and it can be used to verify retention of extracellular proteins. Collagen IV and laminin are ECM proteins that are primary structural components for renal basement membranes.[10] Collagen IV, specifically, is essential for glomerular filtration and has been shown to be important to laminin mediated attachment and migration of murine ES cells.[150] Laminin is a proteoglycan that is involved in a multitude of signal transduction pathways, has been linked to

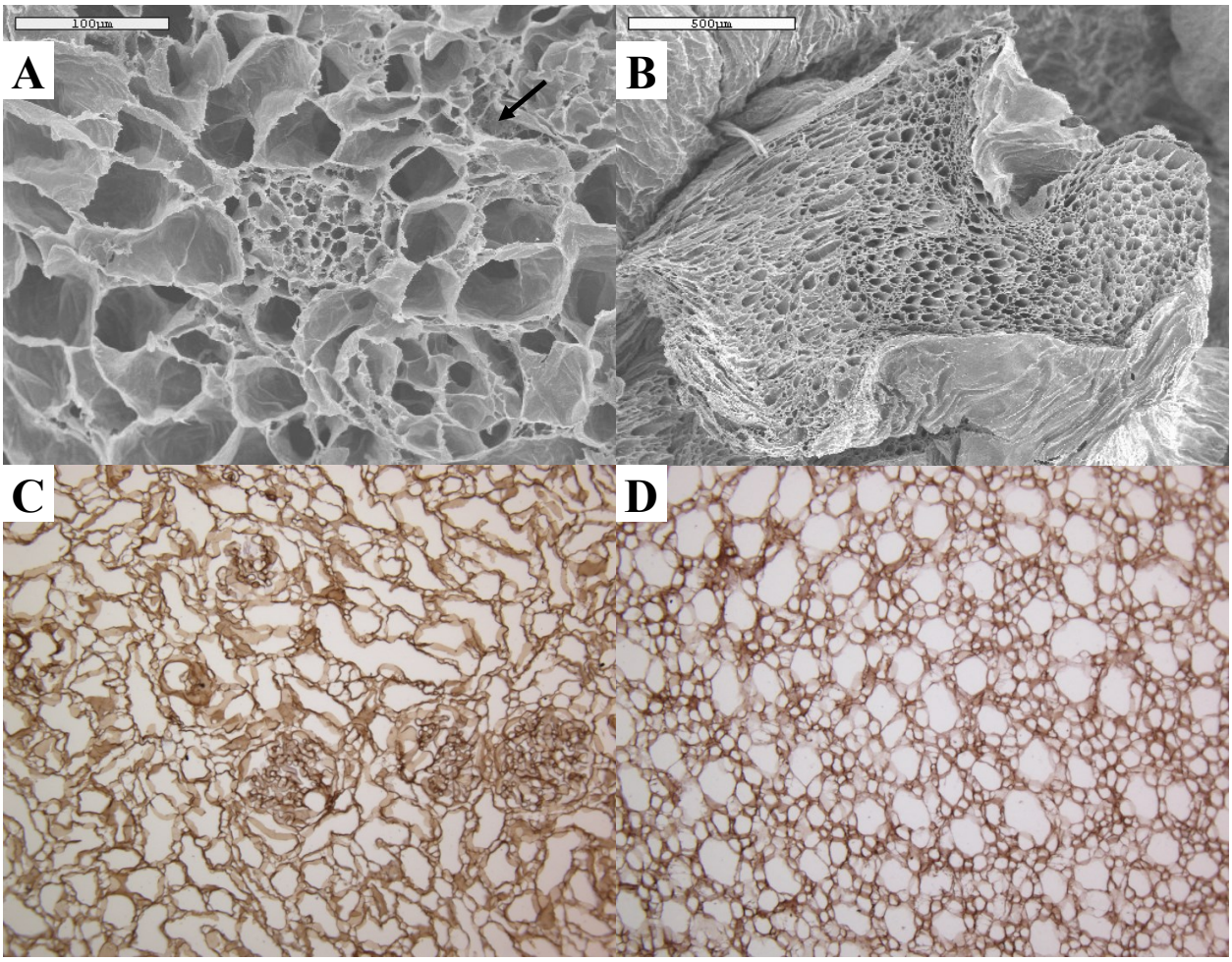

Figure 3-3. Supplementary analysis of decellularized scaffolds. Scanning electron micrographs of acellular kidneys produced with the Graduated 10% protocol, A) glomerulus and adjacent tubules shows continuous basement membrane architecture, arrow identifies cellular debris (300x magnification, bar denotes 100µm), and B) inner medulla (65x magnification, bar denotes 500µm). C-D) IHC stains verifying retention of key basement membrane proteins, C) collagen IV in the cortical region of scaffold and D) laminin in the medulla (*Kidney SDS* protocol, 40x objective magnification).

nephrogenic processes, and has been shown to play an important role in the *in vivo* fate of embryonic stem cells.[10, 51] Collagen and laminin IHC was performed on scaffolds generated using the *Kidney NaDC* and *Kidney SDS* decellularization protocols. As expected, collagen IV and laminin was ubiquitous and continuous over the entire renal scaffold (Figure 3-3). Reference IHC for normal tissue is included in the appendices, Figure B-1. Interestingly, in the scaffolds

produced using the *Kidney NaDC* protocol, the heretofore eosinophilic cell debris appeared highly stained for laminin yet clear of collagen IV (data not included).

Cell Removal Immunohistochemistry

In addition to assuring retention of extracellular matrix proteins, immunohistochemistry has also been utilized to verify the removal of cellular proteins.[20, 22, 24, 109] It offers an additional check beyond the visualized removal of eosinophilic debris through hematoxylin and eosin histochemistry. Further, antibodies to proteins specific to distinct cell populations in the kidney allow identification of those cells or cell lysates which resist removal. In the following chapter, embryonic stem cells implanted and cultured in decellularized scaffolds were tested against four antibodies, pan-cytokeratin, WT1, Pax-2, and Ksp-cadherin to determine potential renal differentiation. The target proteins are also present in the adult kidney including glomerular, tubular, and collecting duct epithelial cells.[151-155] Table 3-2 compares the effectiveness of the two most utilized decellularization protocols, *Kidney NaDC* and *Kidney SDS*, at removing any trace of the indicated cellular proteins. According to immunohistochemical observation, the improved *Kidney SDS* protocol was successful at removing all traces of these proteins. The *Kidney NaDC* protocol, on the other hand, showed retained WT1 localized to the glomeruli; this is consistent with normal podocyte expression. Pax-2 and Ksp-cadherin immunohistochemistry showed positivity in the unwashed cellular debris, which could indicate intact cellular proteins. However, an alternate protein detection technique should be employed to exclude nonspecific binding of the antibodies to the debris. Examples of these two cases, residual cellular proteins in expected location and in cellular debris are displayed in Figure B-2 in the appendices.

Table 3-2. Kidney cellular proteins removed in decellularization process

Protocol	pan CK	WT1	Pax-2	Ksp-cadherin
Kidney NaDC	-	+	D	D
Kidney SDS	-	-	-	-

Based on immunohistochemical observation, the *Kidney SDS* decellularization protocol is more effective than the *Kidney NaDC* protocol at removing cellular proteins in adult rat kidneys. Cytokeratins (pan CK) are intermediate filament proteins present in most renal epithelial cells; WT1 is a transcription factor found in glomerular podocytes; Pax-2 is a transcription factor primarily localized to cells in collecting ducts and descending limbs in loops of Henle; Ksp-cadherin is a kidney-specific transmembrane adhesion protein located in all cells in the nephron except for those in the glomeruli, blood vessels, and papillary epithelium; it is especially pronounced in the distal convoluted tubules and collecting ducts.

Key: +, visualized residual proteins in proximity to expected location in normal kidney; -, no visualized proteins; D, no visualized proteins in expected location, coloration indicates possible diffuse presence in unremoved proteinaceous debris.

Discussion

Tissue Engineering Scaffold

Rat kidneys have been successfully decellularized. This has been achieved through careful organ harvest, design and construction of a perfusion apparatus for decellularization, and the development of a protocol that produced intact acellular organs. The optimal solution sequence was based on the ionic detergent sodium dodecyl sulfate. The utility of the produced acellular kidneys as a renal-specific tissue engineering scaffold, however, remains to be evaluated.

The ideal tissue engineering scaffold is a structure that, once implanted, will not result in a destructive inflammation or immune response and will direct resident or engrafted cells to functional configurations. In the case of such a scaffold being generated from organ decellularization, key parameters for success include the removal of all antigenic or inflammatory elements, the preservation of three dimensional structures, and the retention of biomolecules which support the desired cellular behavior. Unfortunately, most of the steps in a typical decellularization process modify the native organ so as to improve one parameter at the

sacrifice of another. For example, stronger detergents are more effective at cell lysis and debris removal but at the cost of increased solubilization or damage of extracellular matrix proteins.[11, 20, 22, 109] This research utilizes the removal of cellular and nuclear debris, retention of collagen IV and laminin, and preservation of extracellular matrix morphology and ultrastructure by histology and SEM to gauge success. These parameters have been shown in analogous contemporary protocols to improve implanted cell viability, proliferation, and encourage site-specific differentiation.[20, 40, 108, 114] However, defined criteria for specific evaluation of whole organ kidney scaffolds in respect to renal oriented cellular responses have not yet been established. This would require an intensive cyclical process of decellularization protocol modification, acellular kidney production, scaffold characterization, measurement of implanted cell response, and further educated revision of the decellularization protocol. Cell response to the scaffolds generated in this research is discussed in Chapter 4. However, it was beyond the current scope of the project to modify the scaffold generation protocol based on cell response feedback. Nevertheless, considering the results of the decellularization experiments, there are scaffold analysis techniques and protocol modifications that may be considered to improve outcome.

Acellular Kidney Analysis

The characterization of produced renal tissue scaffolds is central to making educated improvements to the decellularization process and for estimating the determinants of engrafted cell behavior. Specific target areas include verification of cell lysis and cell remnant removal, retention of biologically active extracellular matrix and matrix-associated molecules, and preservation of a continuous three dimensional extracellular tissue architecture.

Cell debris removal. One of the primary advantages of using decellularized tissue for an implantable tissue engineering scaffold is the sharp reduction of destructive host immune response as compared to a whole organ transplant.[41, 42] It has been suggested that the cause is

removal of cellular antigens and the conservation of ECM protein structures within and across multiple species.[11] Further, it has been shown that the decellularization process will allow implants from xenograft scaffold sources, easing reliance on human donors.[98, 156]

Figure 3-2 demonstrates a progressive removal of cellular debris from the kidney as observed with H&E histochemistry. In particular, all treatment variants show complete reduction of nuclear hematoxylin-positive staining. Hematoxylin and eosin histochemistry has been used to verify removal of DNA in other decellularization studies; it has the advantage of showing localized areas of poor decellularization in reference to intact extracellular anatomy.[21, 114] Alternately, some groups have used 4',6-diamidino-2-phenylindole (DAPI) fluorescence histochemistry.[20, 25-27] DAPI directly interacts with nucleic acids and can be used on histological slides or live cells. Quantitative methods using DNA isolation and spectrophotometry are also popular for analyzing removal of cellular debris but they are normalized to total tissue mass or total protein and lack the capability for convenient location mapping.[22, 24, 27] Given the importance of DNA removal for graft acceptance, Crapo *et al.* (2011) suggests that H&E alone is comparatively insensitive and that a combination of criteria should be established for removal verification:

- <50 ng dsDNA per mg ECM dry weight
- <200 bp DNA fragment length
- lack of visible nuclear material in tissue sections stained with DAPI or H&E

These criteria are offered as a baseline for constructive host remodeling, yet will need to be verified, modified, and expanded according to specific tissues and implant locations.[108]

The decellularization protocols developed in this research have resulted in a progressive reduction of eosinophilic protein debris in produced scaffolds as visualized by routine H&E histology (Figure 3-2). It has been shown elsewhere that an overall reduction in residual cellular

material results in improved cell growth on implanted scaffolds.[40] In many cases, the tissue surrounding the glomeruli was clean, while the outer medulla was congested with protein aggregation. It could be interpreted that during decellularization the cell lysates from the glomerular capsules and proximal tubules travelled downstream at a rate that exceeded the ability of the more distal tubular pathways to clear. In response, the decellularization protocols were adjusted in attempt to reduce release rate of cellular lysates or improve solubility of aggregated proteins. One caveat to the use of H&E histochemistry is that there is no distinction between cellular or extracellular proteins. Therefore, other contemporary protocols have incorporated techniques to recognize cell specific antigens for verification of cell debris removal.[20, 22, 24, 109] These techniques include IHC, protein immunoblot (western blot), and enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry allows visualization with associated structures while western blot and ELISA provide a level of quantitation. ELISA is the most accurate yet comparatively costly.[157] In this research, IHC analysis was performed on decellularized scaffolds with a small handful of antibodies. These antibodies covered an assortment of cells, including all epithelial cells in the nephron. It showed that the *Kidney SDS* decellularization protocol was clearly superior to *Kidney NaDC* in terms of cell debris removal. The antibodies used also provided some degree of localization information. For example, detection of Ksp-cadherin without Pax-2 may indicate retained cellular proteins from the convoluted tubules or portions of Henle's loop. In other decellularization research, the proteins that have been used to verify cell removal include MHC1 (major histocompatibility complex 1) and β-actin as generic, ubiquitous cellular proteins and CD31 or PECAM-1 (platelet endothelial cell adhesion molecule) as endothelial cell markers.[20, 24] MHC2, a protein that specifically activates host immune response, has been targeted as well.[24] Others have also used markers that

identify the cells specific to the organ.[22, 109] Given the diverse arrangement of cells in the kidney, it may be beneficial during decellularization protocol optimization to use a set of markers that uniquely identifies each of the diverse cell populations. Yabuki *et al.* (2011) suggest all cell types on the kidney can be identified by the binding patterns of a particular group of lectins. Lectins are highly specific sugar binding proteins involved in biological recognition phenomena.[158] Another paper uses a combination of intermediate filament proteins such as individual cytokeratins and vimentin to distinguish normal tissue from renal epithelial tumors; these could also be used to identify each group of kidney cells.[153] The use of these types of marker sets will prove that cell phenotypes have been removed, or if not, which should be targeted. Finally, the SEM images acquired in this study were taken to show a continuous preserved extracellular matrix; however, protein aggregates were also easily recognized (Figure 3-3). Scanning electron microscopy has been to verify cell material removal in other studies, but similar to H&E histochemistry, it lacks ability to distinguish between cellular and basement membrane proteins.[24]

ECM retention. Vascular, glomerular, tubular, and collecting duct basement membranes are specialized extracellular matrices that support adhesion, growth, polarization, and differentiation of the numerous types of epithelial cells that are in the developing and adult kidney.[10, 51] Many of the constituent molecules are directly involved in nephrogenesis and have been shown to direct stem cell differentiation.[30, 51, 159-163] Further, it is thought that native extracellular protein architecture is optimal for organ-specific cell behavior and is the most suited environment for differentiation of stem cells into a targeted tissue phenotype.[18, 30, 159, 164] Primary component proteins of renal basement membranes include collagen IV, laminins, entactin/nidogen, and an assortment of proteoglycans.[10, 46, 51] Methods for protein localization or

quantification can be used to assess the preservation of these essential proteins in decellularized kidney tissue.

Laminin and collagen IV immunohistochemistry was performed on decellularized kidneys (Figure 3-3), and as expected both of these antibodies showed complete and continuous coverage in the tissue scaffold.[10, 47, 165] Interestingly, scaffolds produced with decellularization protocols utilizing NaDC showed the proteinaceous debris as heavily positive for laminin and negative for collagen IV (data not shown). This may indicate that the NaDC protocols cause sloughing of laminin during perfusion, whereas SDS-based protocols do not. Alternatively, it may imply that SDS-based treatments, in which scaffolds are mostly devoid of debris, are actually more effective at solubilization of basement membrane proteins. This data should be considered preliminary, however, until alternative methods of protein quantification can be used for validation, and to rule out unspecific laminin antibody binding or entrapment in the proteinaceous debris. The use of IHC for extracellular protein detection is commonplace in analysis of acellular tissue; however, there is usually little effort expended to identify specific collagen or laminin isoforms.[18, 20, 22, 23, 25, 27, 40, 114] Given the heterogeneous structure of kidney ECM, preservation of each of the isoforms in their native locations may be crucial in establishing functioning tissue and showing this would be a viable continuing step in this research.[10] In addition, methods for quantification of protein loss during decellularization would be valuable. Even though IHC provides location-based information, and even with densitometric analysis, total quantities are difficult to interpret.[166] Some research teams have used a colorimetric assay for hydroxyproline concentration, which would approximately quantify loss of collagen.[24, 26, 103] Others have used western blot.[18, 22] ELISA may also be employed.[157] These techniques are not location-specific, yet perhaps multiple samples of various regions of renal tissue through careful

dissection would yield more focused results. These quantitative measures may be normalized through lyophilized weight or an estimation of total protein.[24]

Recently proteoglycans, or associated glycosaminoglycans (GAGs), have become more common targets of acellular scaffold analyses.[18, 24, 26, 27, 114] Proteoglycans are involved in cell adhesion, growth factor binding and delivery regulation, and are necessary for a multitude of nephrogenic processes.[48, 49, 52-54, 56, 57, 167] They have been shown to be necessary for renal growth and development, and the removal of GAGs and other adhesive proteins slow cellular migration and reduce cell growth on acellular tissue.[51, 105, 149] Key proteoglycans in renal basement membranes are perlecan, agrin, bamacan, and collagen XVIII. All those listed are heparan sulfated proteoglycans (HSPGs) and carry primarily heparan sulfate GAG chains, with the exception of bamacan, a chondroitin sulfate proteoglycan. Heparan sulfate proteoglycans are required for mesenchymal condensation and initiation of branching morphogenesis.[53, 167] Perlecan is in all adult renal basement membranes, improves stability under mechanical stress, and is present in ureteric bud basement membrane.[50, 51] Agrin has a strong presence in GBM, although its role in glomerular filtration isn't clear.[50, 51, 168] Bamacan is present in all adult renal BMs except GBM, although it is also seen in the developing glomerulus.[10] Collagen XVIII plays a role in mechanical integrity of the glomerular and tubular basement membranes, is implicated in Wnt signaling pathways, and is required for ureteric bud branching.[51, 169] The identification and quantification of proteoglycans or specific glycosaminoglycans after the decellularization process would be an informative future addition to this research. One method for GAG quantification is a spectrophotometric approach that uses dimethylene blue dye.[24, 26] This approach can distinguish between chondroitin, dermatan, heparan, and keratan sulfates using polysaccharidases.[170] On the other hand, less quantitative yet location specific information can be

obtained with IHC. Alcian blue is a cationic dye that has been used on decellularized constructs to highlight GAGs.[24] Alternatively, Nakayama *et al.* (2010) used heparan sulfate proteoglycan IHC to observe decellularization-induced changes.[114] Any of these methods would further improve the characterization of acellular kidney scaffolds.

As mentioned, extracellular matrix proteoglycans may sequester growth factors, serve as a delivery reservoir, and act in their regulation. Growth factors stimulate cell proliferation and differentiation and serve roles in ECM protein regulation.[52, 54, 55] Unfortunately, the process of decellularization, especially protocols containing ionic detergent, may cause a sharp reduction in ECM bound growth factors.[11, 70] However, this needs to be verified with the kidney scaffold, especially if GAGs survive the treatment. De Kock *et al.* (2011) detected surviving VEGF in a decellularized liver after both nonionic and ionic detergent perfusion.[21] Nakayama *et al.* (2010) has shown with IHC that HSPGs, while reduced, may survive decellularization of renal tissue.[114] Prepared acellular tissue that is growth factor depleted, yet retains preserved GAGs, may be exposed to growth factors in solution for re-association. Lai *et al.* (2006) has used this technique with bovine pericardia and basic fibroblast growth factor. This "loaded" tissue showed sustained, gradual growth factor release *in vitro* and improved angiogenesis *in vivo*.[171] Growth factors that bind to extracellular proteoglycans and play a role in renal function or development include, among others, VEGF, PDGF, HGF, FGF, TGF-β, BMP-7, and Wnt-4.[52, 54-56] VEGF binds to perlecan and is responsible for renal vasculogenesis, glomerulogenesis, and tubulogenesis as well as endothelial cell differentiation.[56, 172] PDGF modulates endothelial functions and enhances ECM protein synthesis.[52] TGF-β also plays a role in protein ECM protein synthesis.[55, 173] HGF is expressed in the metanephric mesenchyme and regulates tubulogenesis.[52] FGFs interact with the GAG chains of syndecan and perlecan, mediate activation of WT1, are involved in matrix

production, are critical to growth and patterning of all renal lineages, and play roles in early and late stages of development.[174, 175] BMP7 binds to the ECM protein fibrillin-1, plays a role in branching morphogenesis, and mitigates secretion of pro-inflammatory cytokines by tubular endothelial cells.[176, 177] In addition, BMP7 has been specifically shown to differentiate embryoid bodies into intermediate mesoderm according to increased Pax-2 expression and ability of cells to integrate into developing tubular epithelia.[121] Wnt proteins are lipid-modified glycoproteins that are stabilized by HSPGs.[54] Wnt-4, specifically, facilitates tubular regeneration in the adult kidney and is required for tubulogenesis through induction of mesenchymal to epithelial transition.[138, 178] Also, WNT4 with enhancement from HGF and Activin A can differentiate murine embryonic stem (mES) cell into renal tubular cells.[179] The identification of growth factors in decellularized tissue or in modified tissue is typically accomplished with an ELISA assay[149, 157, 171] Immunohistochemistry may also be employed. The preservation or manipulation of growth factors in the decellularized kidney scaffold may be key to directing regeneration and should be in strong consideration for any proceedings studies.

Structural preservation. The kidney has elegant and moderately tortuous three dimensional structures which efficiently direct blood distribution and filtration; metabolite, ion, and water reabsorption; and collection and removal of urinary filtrate. The preservation of native kidney extracellular architectures may play a key role in the generation of newly functional tissue, facilitating the correct anatomical arrangement of populating cells. Other decellularized organ matrices have been used to generate functional organs such as a beating heart, a lung capable of oxygen and carbon dioxide exchange, and a liver that secretes albumin.[22-24, 26] Although each of these tissue engineered organs is still being developed, they are arguably the most advanced solutions yet devised, and have insofar the greatest potential to become clinically

relevant. All of the decellularized organs used to generate these structures have demonstrated intact native extracellular architecture, including functional vascular networks.

The kidney scaffolds produced with this research have retained their overall shape with a visually identifiable vascular tree. In addition, all relevant glomerular, tubular and collecting duct structures remained intact and appeared continuous with expected sizes and segmentation. It has been suggested that preserved morphology improves cell growth in decellularized scaffolds.[40] The morphology of the kidney scaffold was inspected using gross visualization, stained tissue slides, and SEM; however, other avenues for characterization exist that may enhance elucidation. Validation of vascular system integrity could be performed with injected Evans blue dye or contrast for microCT.[23, 24] Considering histology, identification of discontinuities could be improved using specialized dyes that enhance basement membrane contrast such as Periodic acid-Schiff or Jones' Silver.[180] Scanning electron microscopy was used to examine surface topography of the kidney scaffolds. The scaffold selected was produced with a high detergent concentration decellularization protocol with NaDC as the ionic detergent. This is arguably one of the more aggressive treatments with most potential for extracellular matrix damage and it was assumed that this would be a worst case representation. However, the SDS-based protocol, which was superior in debris removal, was never successfully tested so it may be prudent in the future to validate with additional SEM studies. TEM (transmission electron microscopy) is another imaging modality that is typically used to inspect cell morphology and cell-cell and cell-matrix interaction in tissue, often with the use of heavy metal immunolabeling. It has also been used, though, to examine ECM morphology and ultrastructure. For example, TEM has been used to examine lung ECM, verify elastin and collagen fibers in explanted acellular tissue engineered constructs, and inspect collagen bundle size, grouping, and spacing in decellularized porcine

pulmonary valves.[24, 181, 182] Brendel *et al.* (1979) used TEM and perfused particles to examine glomerular filtration properties of decellularized kidneys[141]. It may be beneficial to repeat these experiments with the SDS-derived scaffold to assure that filtration is not diminished and that basement membrane ultrastructure is minimally damaged.

The following chapter details the injection of embryonic stem cells into the kidney scaffold through the renal artery. Initially, the cells remained confined to the glomeruli, likely occupying only the vascular capillaries. It may be, even though there are other routes of cell delivery, that a degree of basement membrane porosity is desired to improve migration of seeded cells into all areas of the kidney. This would require mechanical stress, excessive detergent treatment, or exposure to an ECM degrading enzyme. The tradeoff is such treatment would likely damage the ECM and constituent molecules, reducing effectiveness as a biological scaffold. A phenomenon that occurred in SDS-derived kidney scaffolds seeded with cells is shrinking of overall structure over incubation time (Chapter 4). This did not occur in the scaffolds designed with NaDC ionic detergent. It may be that incomplete removal of cell debris in the NaDC scaffolds provided resistance to scaffold deformation. It may also be that the SDS-based procedures caused a change in ECM composition or component binding. Cortiella *et al.* (2010) saw shrinkage in seeded and incubated lung scaffolds after decellularization with SDS.[20] Nakayama *et al.* (2010), in which a static fluid preparation was used, saw size reduction in their Rhesus monkey kidney scaffolds after treatment with SDS.[114] It may be that perfusion served to delay the effect for the scaffolds presented earlier this chapter. A potential solution is the decellularization of kidneys at low temperature (4ºC), which prevented the scaffold shrinkage in the Nakayama *et al.* protocol.[114]

The kidney scaffolds have endured median physiologic fluid pressures during decellularization without disruption of the ECM. This does not, however, simulate probable *in vivo* conditions, which include compression, shear, and exaggerated fluid pressures. Nakayama *et al.* (2010) showed a significant reduction in compressive modulus in a decellularized kidney after their treatment.[114] In the matter of vasculature, rat iliac arteries were decellularized using SDS and NaDC, yet retained roughly 80% of native vessel stiffness, much better than artificial constructs.[101] Similar mechanical properties in engrafted vessels play a key role in graft success. Mechanical testing should be considered before scaffold implantation, with or without engrafted cells.

Decellularization Protocol

Organ decellularization is typically achieved with perfusion of solutions through the arterial supply. Success is determined based on the effectiveness of the fluid delivery apparatus and the sequence, composition, and exposure duration of the perfusates. This process should ideally be robust to anatomical variation and provide scaffolds that have minimal residual cell material, do not illicit immune response, and support functional tissue growth. Crapo *et al.* (2011) provides a focused review of perfusion-based organ decellularization protocols.[108] Circulated solutions may include detergents or acids to solubilize cell membranes and intracellular contents, chelating agents to disrupt cell attachment, or enzymes such as proteases or nucleases for cell digestion. In addition the solutions may be hypotonic or hypertonic to provide osmotic stress for cell membrane rupture. The act of fluid perfusion naturally applies shear stress to intact cells and provides a means of washing away cell debris. Moreover, supplemental methods for cell disruption have been employed such as freeze-thaw cycles, agitation, and sonication.[11, 20, 27, 108] Highlighting the advantages of perfused solutions for scaffold production, one protocol achieved lung decellularization with arterial and tracheal

perfusion of SDS in 2-3 hours, whereas decellularization in a rotating chamber with freeze/thaw cycles and DNase/RNase treatments required 5 weeks of detergent exposure.[20, 22]

The kidney decellularization protocols in this research primarily utilized non-ionic and ionic perfused detergents with variations in concentration, perfusion duration, sequence, and total number of solutions. Osmotic stress was achieved with distilled water and high salt concentrations. The digestion of nuclear material was facilitated with deoxyribonuclease and the addition of divalent cations for activation of endogenous nucleases. A series of progressive protocol modifications have yielded a decellularized kidney with minimal cell debris, preserved morphology, and retention of primary ECM proteins. However, the process has not been optimized for preservation of other significant basement membrane molecules or reduction in total time, chemical exposure, or number of steps. Such optimization has the potential to improve throughput and reliability and yield a more biologically responsive scaffold.

Organ perfusion. Two distinct perfusion systems were utilized for these decellularization experiments. The first was the LifeSustainer 1000 automated organ perfusion workstation with which the perfusate was driven directly into the organ by a peristaltic pump with either pressure or flow rate control. In pressure control mode, the flow rate of the peristaltic pump was directed by an algorithm with input from a pressure transducer. Unfortunately, two phenomena confounded this control system with our rat kidney decellularization model. First, any air bubbles in the fluid lines, which were difficult to mitigate with the detergent solutions and multiple tubing fixtures, would cause fluctuations in the pressure transducer reading. In addition, these air bubbles had the potential to work their way into the kidney causing multiple emboli. A decellularizing kidney even without these emboli would have increases and decreases in resistance depending on the rate of cell rupture, debris aggregation, and removal of material.

These alone or in synergy were too difficult for the propriety pressure control algorithm to manage without damaging pressure spikes. Unfortunately, even with a constant flow rate setting, air emboli and cell debris caused increases in fluid resistance through the organ and corresponding damaging increases in pressure.

The second perfusion system was an improvement over the first because of the simplification in plumbing and the passive gravity-based pressure control. There were very few areas for bubble generation or entrapment, and the scaffolds did not show any gross damage from excess pressure. A similar system but with fluid height controlled by a glass column was used for kidney decellularization by Brendel *et al.* (1979).[141] Constant pressure decellularization has also been used for heart and lung.[22, 23] Heart perfusion typically employs the Langendorff perfusion technique and the system can be designed with pressure or flow rate control.[183] Pressure control for this technique can also be regulated using a column or elevated reservoir. This comes at the expense of necessary excess fluid volume to achieve the effect. It may be possible to perform decellularization of the kidney using flow rate control if development of air emboli is controlled. It has been successfully used in the liver by a few groups.[21, 24, 25, 27] However, as the kidney has more complicated tubular structures it may require careful monitoring and recording of pressures to assure that a certain flow rate does not cause damage.

One of the benefits of perfusion decellularization, aside from solution delivery and debris removal, is the increased mechanical stress on the cells caused from escalated pressure and shear. Of note, the elevated reservoir perfusion system developed by our lab does not deliver cyclic pulsations to the organs. This is because the peristaltic pump is only used to return solutions to the reservoir, not to drive them through the organ. This was done to minimize potential damage to the ECM that was seen in the previous system. However, it is possible the mechanical

pulsations would be beneficial for improved cell lysis, debris removal, and prevention or disruption of protein aggregation. To test this, the perfusion system may be redesigned to move the peristaltic pump into a fluid driving configuration. A bypass or tube split may be placed between the pump and the organ, allowing "excess" fluid to be pushed up to the reservoir. The magnitude of the pulsations delivered to the tissue will be dependent on the type of pump and the proximity to the organ. This type of system was used for the perfused organ culture detailed in Chapter 6, with a diagram included in Appendix A, Figure A-3.

It may be considered that given a constant pressure, the flow rate of perfusates through a decellularizing organ will change depending on the degree of cell removal, the size of the kidney, and the type of solution. Characteristics such as viscosity, detergency, concentration, and charge may each play a role. Alternatively, the fluid pressure in a decellularizing organ will change over time with a fixed flow rate. While the LifeSustainer 1000 perfusion workstation was in use, it was observed that during constant pressure operation, the flow rate of a perfused detergent tended to increase to a plateau over several hours. Identification of these plateaus for each perfused solution may be a means to optimize duration of exposure. Over the course of the decellularization protocols, the flow rates, measured by drip from the organ, measurably shifted as the solutions changed. Aside from progressive increase in flow as a result of cell removal, the flow rates were generally highest with deionized water, followed by nonionic detergent, and then ionic detergent. Increasing concentration decreased flow rate, as expected. The decellularization of a kidney followed an expected path of flow rate changes; however, more data needs to be collected before it can be used to predict outcome. Insofar, confounding factors may be individual variants in rat kidney anatomy, wide variation in age, and improvement of surgical technique including cannula suturing for leak reduction.

The perfusing organs from both systems were open to the lab environment. A 0.2 micron inline solution filter, addition of the sodium azide antimicrobial to detergent perfusates, and treatment of decellularized kidney with an antimycotic/antibiotic treatment were sufficient to avoid contamination during organ culture. However, a re-design of the perfusion system to assure the components are modular and sterilizable with an isolated organ chamber would further reduce the likelihood of contamination.

Decellularization solutions. Kidney scaffolds have been produced by perfusing a whole organ using a prescribed sequence of solutions. The scaffolds have preserved extracellular matrix structures and basic component proteins with almost complete removal of cellular debris. The predominant measure of success, though, is the ability of the scaffold to influence cells to form functional renal structures. The next chapter details the effects of the kidney scaffold on implanted murine embryonic stem cells. These results may be used as a baseline to gauge further changes to the decellularization protocol. We have shown that increased concentration and exposure duration of perfused detergents or detergent substitutions improve cell debris removal. However, many of these modifications may actually diminish the biological effectiveness of the scaffold by degrading extracellular matrix components and stripping associated growth factors. An optimal scaffold will likely be produced by determining the least aggressive chemical treatments to achieve removal of cell debris, DNA, and other cellular antigens. Ideally, each step of the solution protocol should be evaluated using scaffold characterization and basic response to cell growth.

Detergent selection and perfusion sequence play a key role in the success of a decellularization protocol. In this research, the nonionic detergent Triton X-100 was initially used, with the addition of the ionic detergents NaDC and SDS. Nonionic detergents typically

disrupt lipid-lipid and lipid-protein interactions with preservation of protein-protein interactions. They are effective at solubilizing cytoplasmic membranes and poor at disrupting nuclear membranes. Ionic detergents solubilize both cell membranes but will denature proteins with increased exposure.[11] Many organ decellularization protocols utilize both types of detergents. For kidney decellularization, most of the cytoplasmic and nuclear membranes were disrupted with the use of Triton X-100 with hypotonic and hypertonic solutions. Sodium deoxycholate was added primarily to improve removal of accumulated cell debris. However, the substitution of NaDC with SDS proved to be a much more effective means of debris removal. Triton X-100 has been used to decellularize such tissues as heart valve, coronary artery, and liver, but usually with the addition of proteases or hypotonic solutions.[18, 106, 184] It has been paired with NaDC to decellularize kidney, nerve, and heart.[27, 141, 142, 185] It has been paired with SDS for esophagus, heart, and liver decellularization.[21, 23, 25, 99] In many cases, SDS is called upon to remove nuclei left by a Triton X-100 treatment.[21, 25, 99] In other cases Triton X-100 it is used as a means to wash SDS from the organ.[23] Some treatments of lung, kidney, vein, and amniotic membrane use only SDS, while SDS and NaDC were used for artery and heart valve decellularization.[20, 101, 105, 114, 147, 186, 187] Sodium deoxycholate has been used as the sole detergent for liver decellularization.[188] Triton X-100, SDS, and NaDC have all been used in alone and in combination to decellularize a variety of tissue.

Other detergents or decellularization agents have contributed to success in scaffold preparation and may be considered to improve the kidney scaffold protocols developed herein. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) is a zwitterionic detergent. A zwitterionic detergent is less denaturing than an ionic detergent but has greater solubility than nonionic detergent.[11] It has been used to decellularize lung with >99% DNA

reduction, removal of MHC 1, MHC 2, and β-actin, moderate reduction in elastin (40%) and severe loss of GAGs (10% remaining).[24] Human umbilical artery has been decellularized with CHAPS and SDS and retained good mechanical integrity, yet with a higher tensile modulus or less compliance than native artery.[103] Triton X-200 is an anionic detergent and has been used in nerve decellularization. It was superior to SDS overall, particularly in basic morphology and basil lamina preservation, but not as effective in cell removal. In that same study, Triton X-200 was combined with sulfobetaine-10 and sulfobetaine-16. This theoretically allowed a tighter packing of micelles with improved solubilization and reduction of protein aggregation, ultimately improving non-destructive removal of cell debris. Of the detergent combinations tested, this last one proved optimum for the creation of a nerve scaffold.[109] Tributyl phosphate is a denaturing organic solvent, yet has been shown to have minimal effect on certain ECMs.[11] When utilized for tendon decellularization, it proved to be superior to a sequence of 1% SDS and 1% Triton X-100 detergents in regard to collagen preservation and cell removal.[189]

The selection of which detergents and perfusion sequence are optimal for the generation of a cellular scaffold is likely specific to the organ. A particular example is the use of SDS for tissue decellularization. The reported risk of SDS use is damage to collagen and removal of GAGs, yet this may be mitigated by tissue type.[11, 21, 23, 114, 149] Reing *et al.* (2010) showed marked reduction in growth factors and diminished cell growth after addition of SDS in dermis decellularization.[149] DeKock *et al.* (2010) used SDS to remove nuclei in liver but it resulted in significant loss of VEGF.[21] On the other hand, Ott *et al.* (2008) showed no loss of GAG after decellularization of heart with SDS.[23] Further, Nakayama *et al.* (2010) showed not only that SDS caused no qualitative reduction in HSPGs, but that overall morphology of the renal scaffold was superior to that produced with Triton X-100.[114] From this, it clear that tissues have unique

responses to detergents, thus highlighting the need for organ specific testing and characterization. Further, as in this research, the use of an alternate detergent may yield unexpected and beneficial results.

Aside from substitution or trial of alternate detergents, other steps may be taken to improve the developed kidney decellularization protocol. The final tested protocol iterations used nonionic and ionic detergents with enzymatic and osmotic treatments. Reduction in detergent concentration or exposure duration, complete removal of processing steps, or other modifications have the potential to produce a scaffold with superior cell response. The Brendel and Meezan protocols on which these kidney experiments were based used Triton X-100 concentrations of 0.5% - 3% v/v with suggested sodium deoxycholate concentration of 4% w/v.[141, 142] The protocols in this research primarily adhered to these limits, with the exception of a few trials at up to 10% Triton X-100 concentration. Also, SDS was set to 4% w/v to compare to NaDC at equivalent ionic detergent concentration. It was demonstrated that increased concentration of perfused nonionic detergent (Triton X-100) improved removal of cell debris. Be that as it may, it was further shown that use of SDS improved cell removal more dramatically. Insofar, the detergent exposure for the most effective kidney decellularization protocol *Kidney SDS* is 71 hours for Triton X-100 and 21 hours for SDS. If the use of SDS is the primary determinant of success, then there may be opportunities to reduce both concentration and duration of both detergents in the decellularization protocol. De Kock *et al.* (2011) impressively improved upon a liver decellularization protocol by reducing 72 hours of detergent flush to 1 hour at equivalent concentrations (1% Triton X-100 and 1% SDS). Aside from efficiency in production, this change yielded greater preservation of laminin.[21, 26] Shupe *et al.* (2010) was able to remove residual nuclear membranes in liver with only 0.1% SDS following a graded Triton X-100 series.[25, 27] In

lung, Cortiella *et al.* in a non-perfusion protocol achieved decellularization with a 1% SDS solution, whereas 2% caused excessive ECM degradation.[20] Using perfusion, another protocol successfully decellularized lung with 0.1% SDS at 2 hours duration, although with some residual nuclear material in associated cartilaginous tissue; 0.5% caused ECM degradation.[22] It may be that lower concentration is necessary in a perfused protocol in addition to shortened duration. Whole heart was decellularized using only 1% SDS for 12 hours with a short Triton X-100 rinse. The preparation showed no appreciable reduction in associated GAGs.[23] Nakayama *et al.*, without use of perfusion, decellularized a kidney in 10 days using 1% SDS.[114] With the use of perfusion, and given the success of reduced concentration and duration in other organs, opportunities exist to refine this kidney protocol and minimize time and solution exposure.

One paper suggests that a method to compare detergents in a decellularization protocol is to compare equivalent levels of detergency through the critical micelle concentration (CMC).[109] The CMC is the minimum concentration required for micelle formation. In practice, this value is affected by factors such as salt concentration, temperature, pH, and presence of other surfactants. Also, solubility in ionic detergent is influenced by presence of counterions.[110] Therefore, while a direct comparison between nonionic and ionic detergents based on CMC alone may not be entirely precise, it still serves as a basic reference for comparison. Further, CMC or multiplier may be used as a baseline to test for the lowest effective concentration of detergent. The CMC for SDS is 0.24% w/v and for NaDC is between 0.083 and 0.249% w/v in aqueous solutions. Given the CMC values, the comparison of equivalent concentrations of NaDC and SDS for purposes of decellularization was a feasible approximation. Of note, effective concentrations of SDS for decellularization in other works cited earlier were below 0.24% w/v. Sodium dodecyl

sulfate exhibits a recruiting and cooperative behavior at interfaces, likely allowing it to remain effective at concentrations lower than the CMC value.

The decellularization protocols developed in this research typically required between 7 and 11 solutions steps. Many of these steps have been retained from the foundation kidney decellularization research by Brendel and Meezan (1979, 1980).[141, 142] They designed their original protocols to lyse and remove most of the cellular material without the use of ionic detergent. It included steps to account for detergent gradation, enzymatic DNA removal, additional rinsing, and additives for increasing cell permeability, exerting osmotic pressure, and activation of endogenous nucleases. The theory was that with the long, convoluted tubular pathways in the kidney, the sudden release of cellular lysate and resulting protein aggregation should be avoided; a carefully stepped protocol would mitigate this phenomenon. However, the use of ionic detergent with its improved solubility may obviate these additional steps, as suggested by the preliminary success of the 1-Day SDS protocol.

Successful reduction of decellularization steps will have the advantage of ease of processing, lower cost, and reduction of inconsistencies. In liver decellularization research, two protocols used a gradation of detergents for whole organ decellularization. Shupe *et al.* (2010) used a series of 1%, 2%, 3% Triton X-100 followed by 1% SDS and Uygan *et al.* (2010) used .01%, 0.1%, 1% SDS with a 1% Triton X-100 rinse.[25, 26] This was later improved by De Kock *et al.* (2011) to 1% SDS followed by 1% Triton X-100.[21] This suggests a gradation of concentrations may not be necessary with ionic detergent use. In addition, all these protocols accomplished DNA reduction without the use of DNase. In fact, it has been shown that ionic detergent is effective at DNA reduction, even at a concentration of 0.1% SDS, as confirmed by DAPI and dsDNA quantification.[25, 149] This is with the mentioned caveat of potential growth

factor destruction with ionic detergent use.[149] One lung protocol required use of nucleases following an extended 5 week SDS treatment, but this may have been attributed to use of non-perfused organ methodology.[20] If DNA reduction is necessary for a particular decellularization protocol, and if the scaffold is being subsequently conditioned for cell seeding or implantation, DNA may be effectively reduced by circulation of serum with its associated nucleases.[24, 103] However, this may run the risk of also introducing proteases that are harmful to the matrix.[190] The complete removal of DNase from the kidney decellularization protocols in this research would be a considerable cost saving as well as nullify the risk of lingering enzyme in the produced scaffolds.

The use of deionized water to both exert hypotonic stress on cells and rinse an ECM scaffold is commonplace, but the use of salts to create hypertonic stress is rare in contemporary protocols. The addition of NaCl to detergent solutions in the kidney decellularization protocol by Brendel *et al.* was intended to aid in disruption of nuclear membranes.[141] However, given the effectiveness of the ionic detergent SDS to disrupt cell membranes, this additive and associated steps may not be necessary in the *Kidney SDS* protocol.

Altogether, given the use of the ionic detergent SDS, 8 steps in the *Kidney SDS* protocol are likely not necessary. Recently, whole lung and heart were successfully decellularized using only one ionic and one nonionic detergent perfusion step followed by a pair of rinses, without addition of enzymes or salts.[22, 23] Further, in other research, kidneys were decellularized in a 10-day non-perfused organ process with only SDS and a rinse.[114] This supports the preliminary results of the *1-Day SDS* experiment conducted herein, as perfusion-based decellularization protocols have been considerably less in duration compared to non-perfused counterparts.

Other protocol modifications may improve the kidney decellularization protocols. It has been shown in a nerve decellularization model that use of deionized water caused 30% swelling in the nerve, which was relieved by substitution with phosphate buffered saline (PBS).[109] All of the solutions and rinses in the kidney protocols in this research are based on deionized water, but it may be beneficial to substitute PBS. In other research, liver, lung, and nerve have been successfully decellularized using only isotonic solutions.[24, 25, 109, 147].

It has been theorized that endogenous cellular proteases are a culprit in the variable results of decellularization protocols reported in the literature.[105] Once cells are lysed, these proteases may immediately degrade the ECM. Therefore, some protocols have added protease inhibitors to counter this effect. Typically, ethylenediaminetetraacetic acid (EDTA) is used to inhibit matrix metalloproteinase activity through chelation of metallic divalent cations.[24, 27, 99, 103, 105, 149] Caution must be taken though, as EDTA can also sequester divalent cations, interfering with cell-cell cadherin bonding and cell-matrix integrin-mediated attachment. Premature release of intact cells would likely clog the fluid pathways in the kidney. Decellularization protocols have achieved success by adding EDTA to the detergent solutions.[24, 27, 103, 105, 149] Bhrany *et al.* (2006), on the other hand, only used protease inhibitors during an initial hypertonic cell lysis step; although since they were applied to esophagus, there was no risk of clogging through rapid cell material release. In addition to 5mM EDTA, that group added other protease inhibitors including 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10µM leupeptin, and 1.5µM pepstatin.[99] Relatedly, if DNase or other enzyme is being utilized for decellularization, consideration should be taken as the buffer ions may also activate lysed proteases.

Nakayama *et al.* (2010) performed an informative experiment, testing equivalent decellularization conditions at two different temperatures. They showed that in kidney

decellularization, use of either Triton X-100 or SDS at 4ºC resulted in better preserved ECM morphology than treatment at 37ºC. Further, the scaffolds created using SDS at 37ºC exhibited compaction, signifying loss of ECM integrity.[114] All perfusions used in the research described herein were performed at room temperature, approximately 25ºC. The scaffolds produced using SDS did not initially appear compacted. However, after seeded cell incubation (Chapter 4), the scaffold became noticeably compact; likely due to the stress of cell invasion. Lowering perfusion temperature may provide a solution to this behavior. Other decellularization protocols have used solutions at 37ºC, in some cases to enhance enzyme activity, yet collagen IV has been documented to denature at or slightly below 37ºC.[20, 21, 27, 191] Lowering the temperature of decellularization from 37ºC to 4ºC significantly improved preservation of collagen triple helical structure as tested by circular dichroism spectroscopy.[192]

The final steps in any decellularization protocol is the rinsing of cytotoxic chemicals and sterilization so that the scaffold may be directly implanted or used for *in vitro* cell seeding and culture. The process of rinsing and sterilization often overlap. In this scaffold research, the decellularizing kidneys were exposed to ambient air. However, 0.5% sodium azide was added to the detergent solutions as an antimicrobial and the perfusate passed through a 0.2 micron filter. Further, since the kidney experienced consistent flow, the likelihood of microbe contamination was reduced. Once finished with decellularization, the scaffold was moved into a sterile container and treated under sterile conditions. If earmarked for cell seeding experiments, the scaffold was perfused or pre-treated with serum-free medium. Initial culture medium contained the antibiotics penicillin and streptomycin and a treatment dose of the antimycotic amphotericin B; the latter was reduced to a maintenance dose upon media refresh. Overall, this procedure eliminated contamination for tissue cultures and allowed cells to proliferate, but it would likely

need to be revised for any implantation studies. In recent literature, the rinsing of organ scaffolds that have been subsequently implanted or cultured has spanned from 1 hour to 5 days.[23, 26] Typically, this rinse is PBS.[20, 22-27, 114] Residual decellularization chemicals lingering in the scaffold would be cytotoxic to cells. In this research, the length of the final wash was inherited from previous protocols which were not intended for cell implantation. However, the survival of implanted cells in a prepared kidney scaffold showed the rinse to be sufficient. A straightforward way to optimize this time was demonstrated by Lichtenberg *et al.* (2006), in which time samples of the perfusing rinse were collected and tested in a cellular metabolic assay, MTS.[186] This type of assay exposes cultured cells to the samples and quantifies resulting cell viability and proliferation. Results should indicate the least amount of rinsing time required for complete reduction of toxicity. On a related note, Reing *et al.* (2010) cautions chemicals lingering in the scaffold may interfere with characterization tests. Specifically, unrinsed SDS likely disrupts accurate GAG quantification using the dimethylene blue assay.[149] To improve SDS removal, protocols have followed SDS treatment with nonionic Triton X-100.[23]

Sterilization techniques used for acellular tissue scaffolds vary considerably, and until recently there has been no direct comparison reported.[3, 28, 108] Common sterilization techniques for medical devices tend to denature proteins and generate cytotoxic residue.[28] Notwithstanding, gamma radiation (1.0 - 1.5 Mrad dose) has been used to sterilize kidney and liver scaffolds before implantation.[3, 18] Typically, antibiotics or antimycotics are used for disinfection in the final organ rinse, in storage solutions, or in a media perfusion preceding cell implantation.[20, 22-26, 114] Some protocols have added an antimicrobial such as sodium azide or ammonium hydroxide to the perfusing solutions.[18, 27, 141, 142] Other protocols have incorporated a 0.1% peracetic acid or 4% ethanol flush.[26, 27, 105] Scaffolds treated with peracetic acid have shown preserved mechanical

integrity and good retention of ECM proteins and bound growth factors.[28] In a study of kidney decellularization, Sullivan *et al.* (2012) compared contemporary sterilization methods including gamma radiation and perfusion of 70% ethanol, 0.2% peracetic acid, or SporGon® reagent (7.35% hydrogen peroxide, 0.53% peracetic acid). They found that gamma radiation at 10 Mrad dose was the only method that assured prolonged sterility of porcine scaffolds (out to 6 months) and allowed cell growth.[3] Any reduction in cell proliferation as a result of scaffold irradiation, however, was not assessed. And it remains to be investigated how adversely this sterilization treatment affects somatic cell maintenance or ability to support stem cell differentiation.

Conclusion

A procedure has been developed that used perfusion-based organ decellularization to produce a whole kidney extracellular matrix tissue engineering scaffold. Based on a protocol from Brendel and Meezan developed in 1980, the perfused solutions have been substituted and modified in duration, concentration, and sequence to achieve an acellular kidney with preservation of renal-specific ECM structures, retention of basic extracellular molecules, and thorough removal of cellular debris. This work provides a baseline for further studies in renal scaffold characterization and modification based on *in vitro* cell seed or scaffold implantation studies. The primary challenge is balancing removal of tissue elements that inhibit cell response and retention of elements that promote viability, differentiation, and functional organization. It is likely that the least aggressive protocol to achieve DNA and cell protein removal will yield scaffolds with the greatest potential to direct cells. A straightforward approach is to reduce solutions steps and lower concentration and duration of perfused detergents. Measuring the quantity and distribution of retained GAGs and associated growth factors during this optimization may serve as an intermediate gauge of success. Specifically, promising next steps may include:

1. Incorporation of analysis for residual DNA localization and quantification
2. Measurement of glycosaminoglycan retention
3. Identification of growth factors such as VEGF, PDGF, BMP-7, and Wnt-4 in scaffold
4. Modification of the decellularization protocol to include only SDS at 0.1% to 1%, with Triton X-100 or PBS rinse at 4ºC

Overall, the nature and parameters of the decellularization protocols will evolve as more determinants of cell behavior are recognized and measured and it is ascertained how they are affected by the various techniques and solutions involved in organ decellularization. Further, as these protocols are ultimately intended to produce a medical product, they will likely be influenced by donor species, sterilization technique, recipient species, patient immune status, specific nature of clinical treatment, intended post-process or pre-implant augmentation, federal regulations, industrial process optimization, and market pressures. In the short term, however, and to provide a basic assessment of *in vitro* scaffold functionality, rat scaffolds produced in this research are seeded with mouse embryonic stem cells and cultured. Details of cell response are discussed in the next chapter.

CHAPTER 4
SCAFFOLD CULTURE WITH PLURIPOTENT STEM CELLS

Experimental Plan

Introduction

Whole organ extracellular matrices have been isolated from kidneys through perfusion of detergent-based solutions. It is hypothesized that these matrices will best support the cells and complex tissue structures in the kidney as synthetic cellular scaffolds cannot yet mimic the histoarchitecture of natural intact renal extracellular matrix. The prepared matrices have been characterized by removal of cellular debris, preservation of renal structures, and retention of basement membrane proteins as these parameters have been shown in other models to influence seeded cell growth, differentiation, and organ-specific arrangement. Ultimately, though, utility of a tissue scaffold must be determined through direct examination with a relevant cell population.

Kidney ECM has distinct structure and protein composition within the vasculature, glomeruli, and various segments in the tubules and collecting ducts that support a multitude of epithelial cell populations.[10, 51] Further, it has been postulated that this unique heterogeneous composition plays a role in specificity of the numerous cells in the kidney.[10] Maeshima *et al*. (2006) have demonstrated that adult tubule stem cells cultured with ECM components laminin, collagen I, collagen IV, or fibronectin showed distinct morphologies, attachment tendencies, and grouping patterns.[36] Extracellular matrix produced from a particular organ improves maintenance of resident cells in culture, amplifies growth rate, reduces apoptosis, and guides tissue-specific differentiation.[20, 24, 30, 159, 164]

Decellularized organ ECM has been used on several accounts as a cellular scaffold with intention to reform organs. Notable efforts include liver, lung, and heart. The resulting cell-matrix constructs each gained a degree of functionality such as contraction in the heart, gas

exchange in the lung, and albumin secretion, urea synthesis, and cytochrome P450 expression (a key metabolic enzyme) in the liver.[18, 22-24, 26, 28] However, in these models, all but the liver used fetal or neonatal organs as a cell source. The liver construct was able to use primary hepatocytes, which make up a majority of the mass of a normal liver and carry inherent regenerative potential. The use of same species fetal and neonatal cells offers proof that developing cells of an organ respond to adult ECM, but these sources are not ethically viable as a long term solution. Pluripotent stem cells may serve as an alternate source as they may be derived from autologous adult tissue. The first step is to test the ability of seeded stem cells to respond to the decellularized kidney scaffold; this includes attachment, proliferation, differentiation and alignment of the cells with the preserved structural boundaries of the ECM.

Pluripotent stem cells have the ability to form any cell in the kidney and are highly proliferative.[31-33] Embryonic stem cells, in particular, are readily available from established cell lines, easily maintained, and have an extensive history of differentiation research. Further, differentiation tendencies of ES cells are likely translatable to patient-derived IPSCs for future applications in the clinic.[122] In regard to kidney differentiation, implanted ES cells, through embryoid body intermediaries, spontaneously formed teratomas with rudimentary ductal structures; the cells in these structures expressed markers observed during kidney development.[119] Whole organ kidney extracellular matrix may guide these cells into more advanced renal structures.

It has been shown that differentiation of ES cells is influenced by ECM composition.[160] Moreover, ECM derived from an organ will promote organ-specific lineage differentiation.[18, 20, 30, 159] In renal organogenesis, critical steps are modulated by renal extracellular matrix proteins.[10, 51, 57, 162] These proteins may also promote differentiation events in renal structures derived from

ES cells. After decellularization treatments, the kidney extracellular matrix scaffold showed preserved collagen IV and laminin. Laminin plays a role in branching morphogenesis, polarization of epithelia, and other developmental events, and collagen IV is important to laminin-mediated attachment and migration of ES cells.[51, 57, 150] Insofar, there is no recorded case of ES cell differentiation on ECM specifically derived from the kidney.

In potential future clinical application, whole organ decellularized scaffolds would be produced from those human kidneys unsuitable for transplant, but with intact extracellular matrix. However, successful use of xenograft ECM, such as from pig, would drastically reduce the high demand for human donor kidneys. Certain cell-free preparations of non-kidney xenografts have already been successfully implanted generating a constructive modeling response without eliciting cell-mediated implant rejection.[11, 16, 40, 98, 102] Alternatively, xenogenic whole organ liver ECM was used *in vitro* to maintain functional hepatocytes over 45 days.[193] Biocompatibility and cellular response to xeno-derived scaffolds may be due to the high level of extracellular matrix protein conservation across species.[11] Examination and classification of the response of murine ES cells cultured in rat decellularized kidneys will provide valuable insight into the baseline potential of xenogenic whole organ extracellular matrix to guide proliferation and differentiation of pluripotent stem cells. In the remainder of this chapter, details of these experiments, a presentation and interpretation of results, and promising avenues for continued research are provided. Specific highlights include static and perfusion culture methods, a survey of cell morphology, proliferation, and apoptosis patterns in relation to basement membrane structures, and detection of differentiation markers in comparison with embryoid bodies cultured without exogenous ECM contact. Detailed methods and materials are described in Chapter 6.

Cell Delivery and Scaffold Culture

In a published decellularized liver model, the whole organ ECM scaffolds were effectively populated with hepatocytes via portal vein injection.[26] The hepatocytes initially surrounded the vasculature, but after two days of culture were shown distributed throughout the matrix. Decellularized kidneys from this research have also retained their overall organ shape with intact vascular networks, and so in an effort to repopulate the kidney scaffold and gauge response of mouse pluripotent stem cells to the rat kidney ECM, ES cells were injected into the renal artery and cultured.

The murine ES cells (B5/EGFP) injected into the kidney scaffolds were gifted from Andras Nagy. These cells were designed to express green fluorescent protein (GFP) regardless of differentiated phenotype.[194] This allowed continuous observation during culture relative to the translucent kidney scaffold. The mES cells were maintained in an undifferentiated state using mouse leukemia inhibitory factor (LIF). Before injection, these cells were suspended in a basic differentiation media formulation including 20% fetal bovine serum (FBS), an antibiotic, and an antimycotic. In other research, embryoid bodies differentiated in media with FBS have spontaneously expressed genes essential for kidney development.[119, 195] In attempt to isolate cell response to ECM exposure, no other agents previously proven to promote renal-specific differentiation were added. Once decellularization treatments were complete, the scaffolds intended for injection were briefly perfused with serum-free media and transferred to a biological hood. Then, a suspension of $2x10^6$ mES cells in differentiation media was carefully injected into each renal artery. After delivery, the arterial cannulae were detached and the seeded organs were submerged in conical 50ml tubes filled with differentiation media. The organs were cultured up to 14 days, with media refresh every 2-3 days. That duration was chosen to capture significant milestones if differentiation progressed in time along normal developmental pathways. Murine

embryonic stem cells are typically extracted from the blastocyst at E3.5 (days post coitum).[116, 117] Murine renal development starts at E7.5 with metanephric development initiating at E11, and fully differentiated structures appearing at E17.[48, 196, 197] Finally, it was assumed that there would be insufficient cells at 14 days of culture to cause issue with nutrient or metabolic waste diffusion, something that would be a concern with a full-thickness organ.[198]

The selection of which prepared scaffold and associated decellularization protocol was used for these cell seeding experiments was dependent upon the stage of concurrent kidney decellularization trials. Finally, as these experiments progressed, alternate methods of scaffold culture and cell delivery were performed as described in the forthcoming results.

Cultured Scaffold Analysis

The techniques for analysis of the cultured scaffolds were designed to assess cell distribution and reveal any morphological changes associated with extracellular matrix contact. Throughout culture, gross localization and grouping patterns were actively monitored with fluorescence microscopy. After completion of the culture period, the cell-matrix constructs were fixed, paraffin embedded, and examined using H&E histochemistry. Compared to frozen tissue embedding, paraffin embedding is superior for preservation of extracellular structures. Typically, a pair of kidneys were produced in each decellularization run, with one seeded and another serving as an acellular control. Upon observing the histology slides, localization of cells in relation to the observable structures such as glomeruli, Bowman's capsules, arterioles, tubules, and interstitial spaces were noted. In addition any morphological cell patterns in relation to those structures was also described. After initial assessments, subsequent seeded scaffold experiments were conducted, and basic patterns of cell division, apoptosis, and differentiation were examined to provide a baseline analysis of mES cell interaction with whole kidney extracellular matrix scaffolds.

Results

Whole Scaffold Culture

Cells that were injected into the renal artery of whole acellular scaffolds achieved significant dispersal with radial streaks, scattered clumps, and appreciable accumulation in the glomeruli (Figure 4-1). Specific localization outside of the glomeruli was difficult to interpret given the uniform appearance of the tubular cavities. At ten days of culture, fluorescent images showed the outline of apparent intact tubular networks. Histology at this time point revealed cells in the glomeruli, within arteries, and interstitial spaces, especially prevalent near the surface of the scaffold. Arteries were identified by the presence of a thicker basement membrane. The decellularized kidney displayed in the figure at culture day 10 received an anomalously poor decellularization for the *Graduated 10%* protocol. However, as the eosinophilic cell debris is most likely aggregated in the tubules, it aided in highlighting the cells as localized to the interstitium. This assertion supports the appearance of the fluorescence images, which shows thinner GFP-positive outlines of larger non-cell-occupied spaces, the tubules or collecting ducts. Further, if the vascular basement membranes remained intact, a portion of the injected cells were likely forced into the interstitial capillary system. Also noticeable at the day 10 time point is the apparent presence of apoptotic cells, primarily in the glomeruli. They are recognized by nucleus fragmentation, small scattered apoptotic bodies, and loss of hematoxylin stain. One last analysis performed on this sample was use of anti-GFP IHC to prove that only implanted cells remained in the scaffold (data not shown). All recognizable intact cells were positive for GFP. One subsequent experimental alteration, after consideration of prominent apoptotic cell presence, was to integrate a perfused media circuit for improved cell viability.

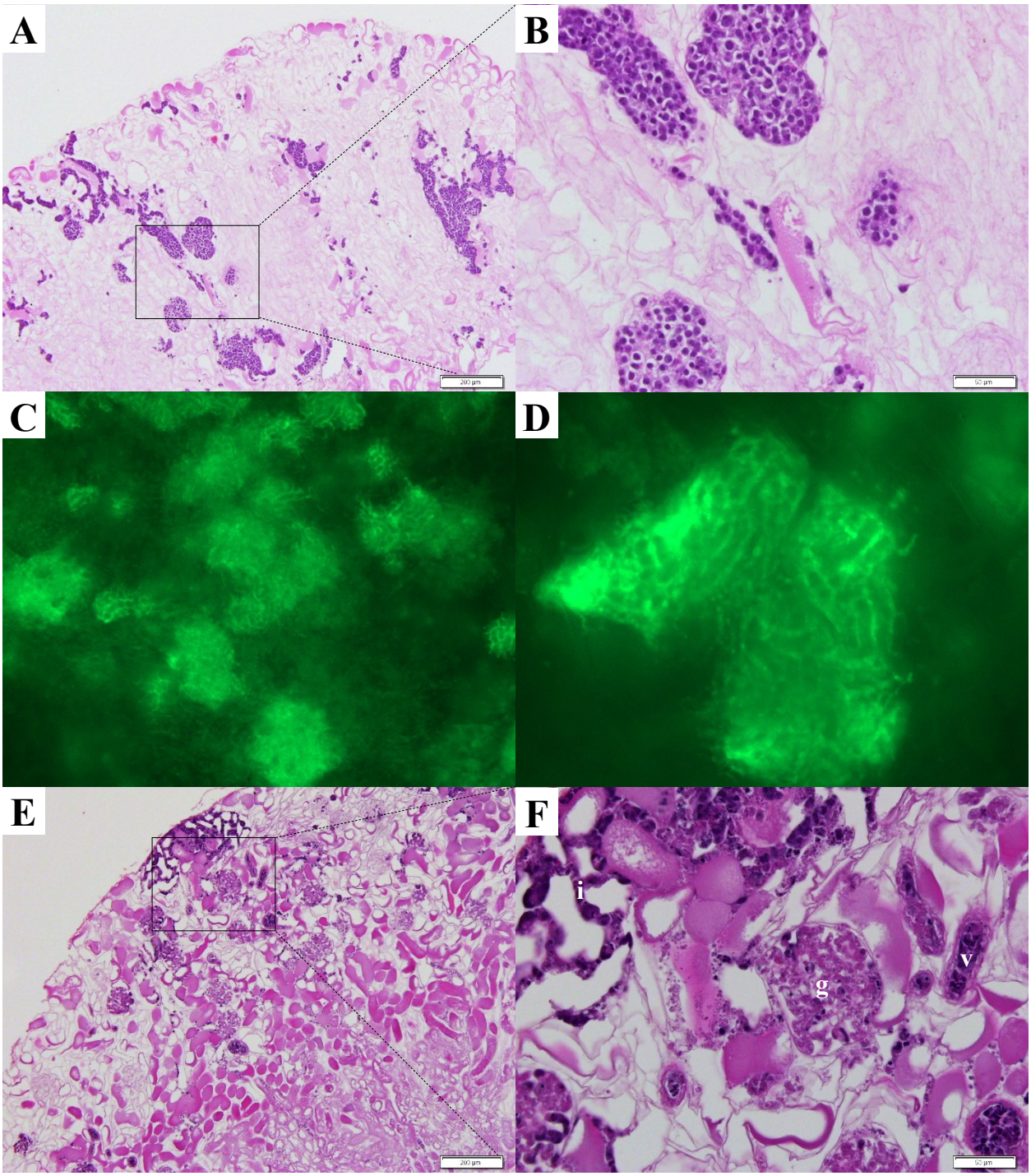

Figure 4-1. Whole scaffold static culture: kidney scaffolds produced with the *Graduated 10%* decellularization protocol and cultured with arterially-delivered B5/EGFP ES murine embryonic stem cells. Cells at 1 day of culture show A) initial cortical distribution and B) filling of glomeruli (H&E stains). C-D) Strong GFP signal from scaffold at 10 days culture suggesting profile of tubular network. E-F) Cells at 10 days of culture shows cells in (v)essels, (g)lomeruli and (t)ubular interstitium (H&E stains). Magnifications: A, D, and E: 10x; B and F: 40x; C: 4x. Scale bars: A and E: 200µm; B and F: 50 µm.

Perfused Scaffold Culture

It is commonly accepted that optimal conditions for generation of an *in vitro* organ or tissue are those that best approximate the natural, healthy *in vivo* environment.[198] In recent literature, there are several notable examples of moving beyond simple static culture to grow organ structures, and the primary mechanism is perfusion of media through the organ.[18, 22-24, 26] This has the advantages of improving gas diffusion and nutrient delivery, and removal of cellular waste[198]. In addition, the perfusion mechanism can be designed to deliver physiologic flow conditions including a pulsatile pressure waveform that mimics normal cardiac output.[23] The simulation of organ-based fluid flow has resulted in improved endothelial and epithelial cell adhesion, viability, alignment and morphological appearance, and improved proliferation and differentiation of organ-specific functional cell types.[22-24, 199]

The decellularized kidneys in this research retained intact vascular networks, and were therefore well-suited to enact media perfusion through the renal artery. If diffusional barriers existed in whole kidney static culture, improved nutrient and oxygen delivery and waste removal had the potential to reduce apoptosis of the seeded embryonic stem cells.[23, 198] Perfused media also has been shown to significantly increase the volume of viable cell structures in seeded scaffolds.[23] Another primary reason for implementing a perfusion culture system is better approximation of the *in vivo* renal environment, as mechanotransduction plays a significant role in kidney function. For example, fluid-based shear stress plays a part in ion movement regulation, stability of fluid reabsorption, secretion of calcium and potassium, and release of nitric oxide for vasodilation.[200] Fluid flow alters epithelial cell gene expression, growth factor synthesis in endothelial cells, prompts realignment of cytoskeletal elements, and modulates tight junction formation.[201] Further, it promotes proliferation and differentiation of stem cells, including ES cells.[201, 202] One specific example is ES cells that express the vascular endothelial

growth factor receptor, Flk-1, are induced to endothelial differentiation with applied shear stress.[202]

Perfusion bioreactor design. A system was designed to maintain perfusing media through a cell-seeded acellular kidney in an isolated and sterile environment. A diagram and images of the apparatus are given in Appendix A (Figures A-3 and A-4). Media was driven by a peristaltic pump. A pressure limiting circuit allowed the pump to be set at a range of speeds without increase in pressure. This was achieved by allowing a majority of the perfusing media to bypass the kidney and travel up a vertical column. The adjustable height of this column set median pressure in the kidney, analogous to the elevated reservoir used in the decellularization apparatus. In addition, increases in resistance caused by cell proliferation would not cause increases in pressure but a compensatory reduction in flow. Close proximity of the pump to the kidney preserved transmission of the pump-generated pulsatile waveform in the media, approximating natural circulation. A mean pressure of 100 mmHg with a 20-30 mmHg pulse range and a periodicity of 270 to 300 beats/min could be achieved, an approximation of normal adult rat circulatory pressure and pulse. Organ and media reservoir chambers were contained in an incubator to regulate temperature. Flow of biomedical grade 5% CO_2 gas through the kidney drip chamber and reservoir chamber was designed to improve regulation of dissolved gases in media. The tubing, connectors, and bottles were all reusable and sterilizable, and most of the plumbing could be assembled in a laminar flow hood. Only a couple of connections were required outside the hood, minimizing the chance for contamination.

Perfused culture results. Decellularized rat kidneys were injected with $2x10^6$ mES cells and sealed within the organ drip chamber of the perfusion bioreactor. After 12 hours to allow for cell adhesion, circulation was initiated. Pressure was only increased to 40/20 mmHg as fetal

aortic blood pressure is significantly reduced compared to normal adult blood pressure.[203] At 3 days of culture, cells were primarily localized to the glomeruli, with limited radial streaks in the medulla (Figure 4-2); total cell numbers were remarkably reduced as compared with a static seed at 1-day (Figure 4-1). The cells at perfusion day 3, however, did not appear inordinately apoptotic, therefore two phenomena may have contributed to the sparse cellularity. First, cells were dislodged from the scaffold once circulation was initiated and pushed through the venous system or if the glomerular basement membrane was inconsistent, passed out the tubules and resulting urinary outflow. Second, the application of shear stress to the mES cells may have served to inhibit proliferation. At day 6 of circulation (Figure 4-2), fluorescence micrographs reveal three distinct patterns in the scaffold, one resembling a glomerular tuft with adjacent vasculature, one an interstitial pattern similar to that observed in whole organ culture (Figure 4-1), and one a pattern resembling filled convoluted tubules. Histology shows glomerular cells with adjacent eosinophilic debris and what appears to be clusters of filled tubules. It is likely that the tubule-pattern noted in the fluorescence images represent the tubule clusters observed in the H&E images. Perfusion of medium may assist the cells in localizing into the tubule spaces. Overall, in comparison with whole organ static culture, perfusion of media through a seeded scaffold results in a sharp reduction of mES cells, but likely improved movement of cells into the tubules.

Sectioned Scaffold Culture

One of the goals of this research was to assess patterns of proliferation, apoptosis, and differentiation of the mES cells cultured on acellular kidney scaffolds. However, until development of the *Kidney SDS* decellularization protocol, quality of cell removal had a greater degree of organ to organ variability. In order to best assess the time course of cellular response, a third method of culture was established. Once a whole organ scaffold was seeded arterially, it

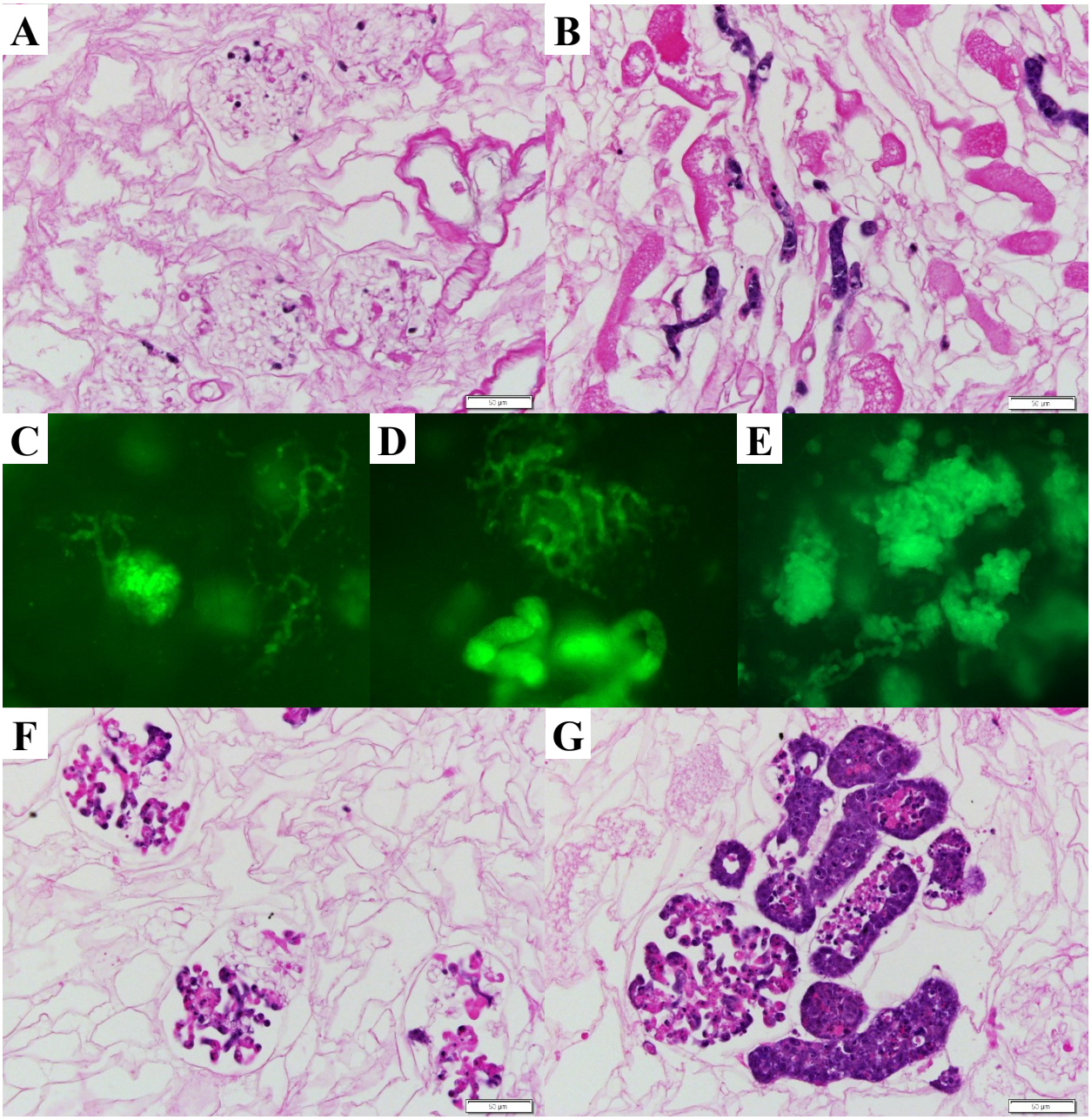

Figure 4-2. Perfused organ culture: murine B5/EGFP ES cells injected arterially into intact kidney scaffold and cultured with perfused media system (*Kidney NaDC* decellularization protocol). Media was continuously delivered through the renal artery. At 3 days, cells are A) dispersed throughout glomeruli with B) some streaks in the medulla at overall reduced number compared to static culture (H&E stains). Fluorescence observation at day 6 shows C) glomerular profile with adjacent vessels, D) two distinct patterns, one interstitial and one tubular, and E) an extensive tubular network. H&E stains at day 6 showing F) glomerular cells with increased eosinophilic debris and G) tubules adjacent to glomeruli filled with cells. Magnifications: A, B, F, and G: 40x; C and D: 15x; E: 4x. Scale bars: A, B, F, and G: 50 µm.

was placed in static culture for a day before being divided into 4-6 thick transverse sections. These sections were cultured collectively in a multi-well dish, with individual sections removed at prescribed time points. The primary advantage to this culture method is exposure of mES cells to identical scaffolds for multiple time points and each section had enough depth to preserve 3D architecture.

Acellular kidneys produced with both the *Kidney NaDC* and *Kidney SDS* protocols were cultured using the sectioned scaffold method and there were many similarities in overall cell distribution and morphology. However, given that superior scaffolds were prepared using the *Kidney SDS* protocol, it is the culture of those that will be the focus of the remaining results. Nevertheless, any differences between the two types will be briefly noted in context.

Cell distribution and morphological patterns. One advantage of using sectioned scaffold culture is the ability to observe a transverse view of the kidney. This allows visualization of seeded GFP positive cells relative to the cortex and medulla as opposed to a simple surface view. As such, over the course of a 14 day culture period, there were more recognizable patterns in gross cell distribution (Figure 4-3). A day after injection into a *Kidney SDS* produced acellular kidney, fluorescence microscopy showed cells heavily localized to the glomeruli. At day 3 and 4 they expanded into adjacent vasculature and progressed into the medulla. After day 6, cells were apparent in the larger vessels. At later time points, day 10 and 14, the cortex appeared increasingly crowded with large clusters of GFP positive signal. In addition, the scaffolds appeared to become more compact. In contrast, seeded scaffolds produced with the *Kidney NaDC* protocol retained a predominantly interstitial cell-occupied pattern as seen in previous whole and perfused organ culture experiments and these scaffolds resisted compaction.

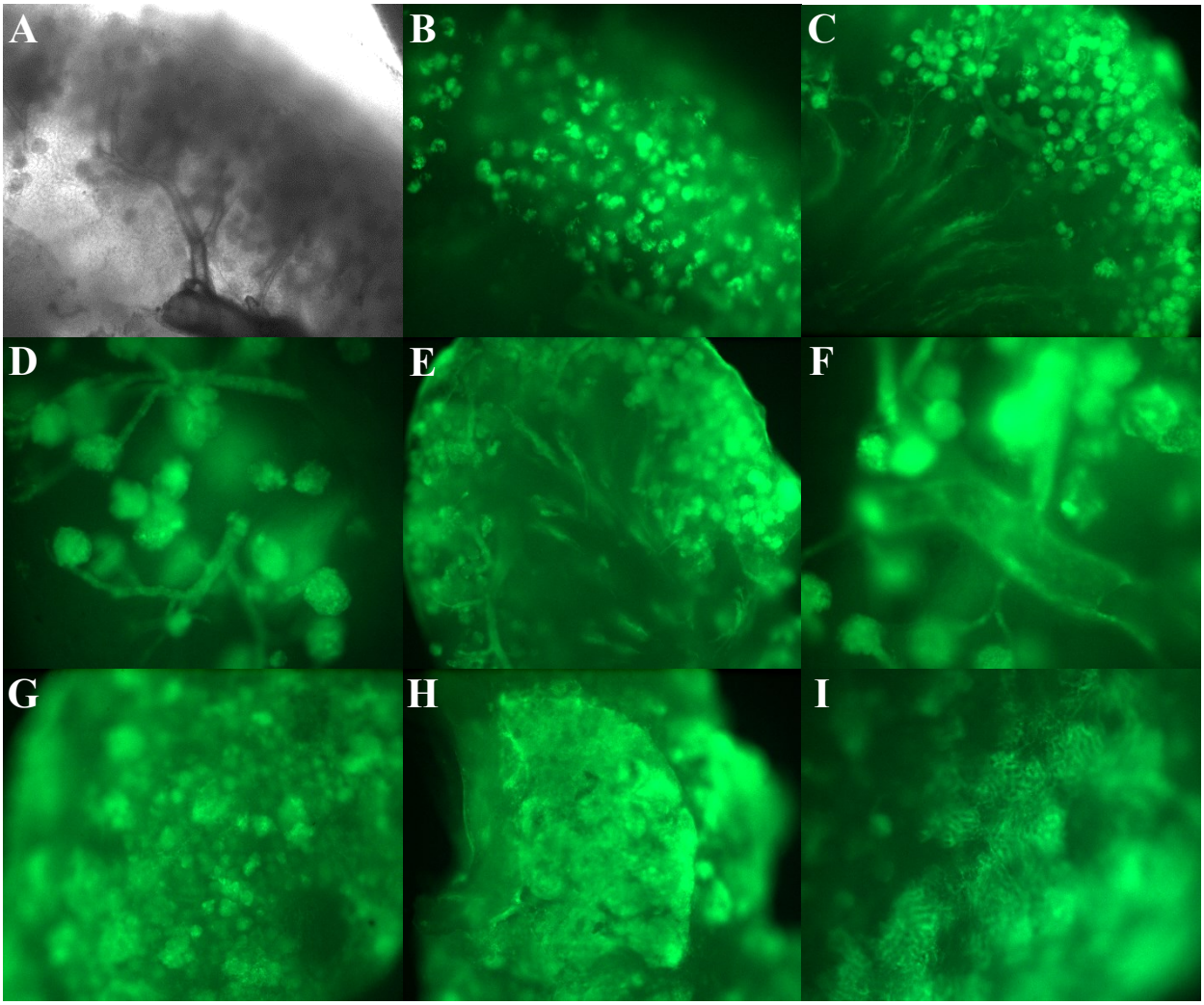

Figure 4-3. Sectioned scaffold culture: typical distribution patterns of GFP-labeled murine ES cells delivered into the renal artery of decellularized kidney scaffolds and cultured for 14 days. Seeded scaffolds were sectioned into 4-6 transverse segments after one day of whole organ culture. A-H) Scaffold produced using Kidney SDS protocol. A-B) Overlapping images of ES cells at 1 day culture showing initial glomerular localization, A) bright field micrograph and B) fluorescence micrograph. C) Outgrowth of cells into adjacent vasculature at 3 days, and D) joining clusters of glomeruli at day 4. E-F) Movement of cells into larger vessels at day 6. G-H) Increasing surface fluorescence and graft compaction at days 10 and 14. I) In comparison, cells cultured in scaffolds produced using *Kidney NaDC* protocol showed similar progression yet with appearance of more fluorescence in interstitial reticular patterning as shown at day 10. Additionally, there is no compaction in these samples. Magnifications: A, B, C, E, G, H, and I: 4x; D and F: 10x.

Once cultures were completed, H&E histochemistry was used to verify cell localization and examine condition and morphology relative to adjacent ECM, including glomerular, tubular,

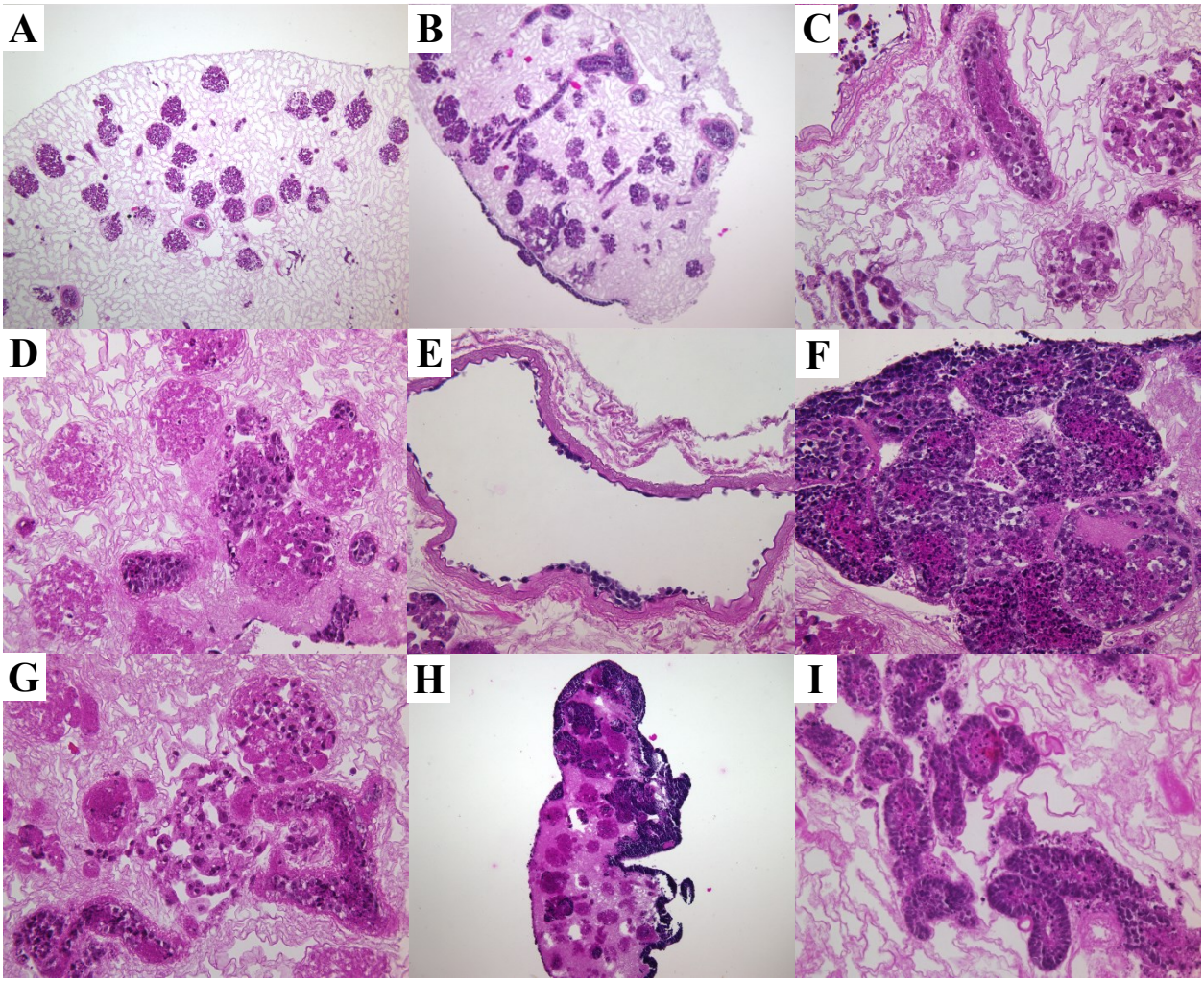

Figure 4-4. Sectioned scaffold culture: morphological patterns of B5/EGFP ES cell growth in acellular kidney scaffolds, H&E stains. A-H) Scaffolds produced with the *Kidney SDS* decellularization protocol. A) At day 4, cells are primarily localized to the glomeruli. B) At day 6, cells pack into the larger vessels. C) Example of intact cells at day 8 in the glomeruli, interstitium, and larger vessels; note the eosinophilic cell remnants in one vessel interior. At day 10, D) most cells are degraded in glomeruli, yet others line E) large vessels and F) expand into tubules. The tubules show patches of deteriorating cells with fractioned nuclei. G) At day 14, cells may show a less crowded spacing in glomeruli and interstitium. H) A severe example of cell overgrowth and scaffold compaction that may be seen with scaffolds produced using the *Kidney SDS* protocol. I) Cells cultured on a scaffold generated with the *Kidney NaDC* protocol at day 10 show more restrained growth in tubules with morphology suggesting luminal apoptosis. Magnifications: A, B, and H: 10x; C, D, E, F, G, and I: 40x.

vascular, and interstitial basement membranes (Figure 4-4). With some variations in cell dispersal and culture time, a typical chronology of cell interaction was observed. In scaffolds

produced with the *Kidney SDS* decellularization protocol, injected mES cells were initially captured in the glomeruli. At 3 or 4 days of culture the cells expanded into adjacent vessels, including peritubular interstitium, and were present in groups as deep as the papilla. Cell presence in tubules was rare at these time points. At days 6 and 8, cells packed into larger vessels, spread on the capsule, and occupied some tubules. Signs of apoptosis were apparent in all locations, but especially so in the glomeruli. At days 10 and 14, cells at various locations, interstitium, tubules, and vessels continued to expand while the scaffold experienced overall contraction. Cells spread out in larger vessels and assumed a squamous morphology. The capsular cell layer thickened and protruded away from the scaffold. Evidence of apoptosis remained, markedly so on the interior of larger packed spaces, such as within vessels or tubules; most glomeruli at this point were filled with eosinophilic debris. However, in these and other areas of accumulated apoptotic cell remnants such as interstitium and tubules, some cells survived in less-crowded configurations. In comparison, cells cultured on *Kidney NaDC* produced scaffolds showed more restrained cell expansion, greater confinement within extracellular matrix boundaries, and less penetration of the interior. Also, the scaffold compression at later time points was minimal.

Retrograde seeding. Delivery through the renal artery of decellularized kidney scaffolds exposed the cells to glomerular, tubular, and vascular basement membranes as well as interstitium in the cortex and medulla. However, population of the tubules was an uncommon occurrence, and usually was not apparent until several days of culture. Retrograde seeding for the kidney involves injection of cells into the ureter. The ureter dilates into the renal pelvis, an open chamber on the interior of the kidney.[4] The pelvis divides into calyces. Calyces surround the apices of renal papillae, pyramidal structures with numerous pores from which collecting ducts

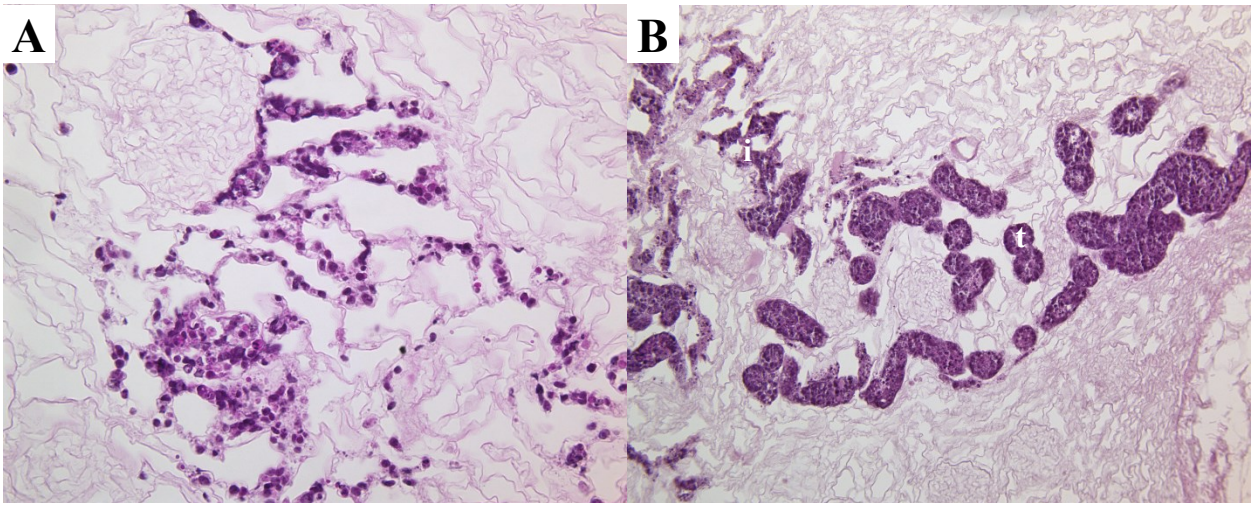

Figure 4-5. Sectioned scaffold culture: cultured scaffold after ureteral retrograde seeding of B5/EGFP cells, *Kidney SDS* decellularization protocol, H&E stains. A) 1 day after seeding, injected cells primarily localized to inter-tubule spaces (40x magnification). B) Cells after 6 days of culture fill both (i)nterstitial space and (t)ubules (20x magnification).

empty. The intention of retrograde seeding was for cells to be driven into the calyces and find their way into collecting ducts and adjoining tubules, improving frequency and distribution of cell delivery to these locations.

Murine ES cells were injected into four acellular kidneys produced with the *Kidney SDS* protocol and cultured 1, 6 and 10 days using the sectioned scaffold method. Only 55% (SD=23%) of $2x10^6$ cells were retained after injection, as opposed to 97% (SD=4%) for arterial injected scaffolds. After a day of culture, it was apparent that distribution of cells throughout the scaffold was patchy and inconsistent. However, cells were present in both medulla and cortex, and in some instances reached the renal corpuscles (Figure 4-5). Interestingly, a majority of the cells seemed to be located in the interstitium, with some suspected to be in tubules. Cells also were noted on the calyceal boundaries. At days 6 and 10 of culture, cells filled tubules and interstitial spaces. In the calyces, cells spread and formed a layer with squamous morphology that thickened over time. No cells were present at any time within the renal corpuscles or arterioles. Overall, this seeding route did deliver a greater relative portion of cells into the

tubules, yet the overall quantity and dispersion of cells penetrating into the cortex and medulla was significantly reduced.

Cell Division and Apoptosis. According to H&E histochemistry, over a 14 day culture period, mES cells seeded into decellularized kidneys appeared to undergo rapid cell division and apoptosis in patterns throughout the scaffold. KI-67 and cleaved caspase-3 (CC3) immunohistochemistry was used to verify these observations and discern specific relationships to glomerular, vascular, tubular, and interstitial extracellular matrix. The KI-67 protein is present in cells undergoing any stage of mitosis whereas CC3 is present in cells that have initiated apoptosis.[204, 205] At day 3 of cell culture in a *Kidney SDS* scaffold there was a strong presence of KI-67 in glomeruli, interstitium, and arterioles, yet CC3 was also showing in these areas (Figure 4-6). At day 6 of culture, cell division was present in all areas as noted at day 3, as well is in tubules. Interestingly, KI-67 appeared strongest on cells adjacent to arteriole basement membrane, within expanding tubule cavities, and spreading on the capsule. Cleaved caspase-3 was also present at all locations, yet reduced in the vessel interior and on the capsule. Glomeruli at this time were heavily apoptotic, and positivity persisted in these locations beyond destruction of cells. At later time points, such as day 14, the marker for cell division remained in scattered cells. These cells were not in aggregated groups as at earlier culture times. In addition these cells thrived even in proximity to large fields of apoptotic residue. Also notable at this culture time are cells which appear quiescent, with no indication of cell division or apoptosis.

Differentiation. In order for stem cells to effectively repopulate kidney scaffolds, it is necessary for the scaffolds and culture conditions to promote differentiation of those cells into functional renal phenotypes. In these experiments, the cell culture medium has not been specifically designed to promote renal differentiation so the ability of the scaffolds to promote

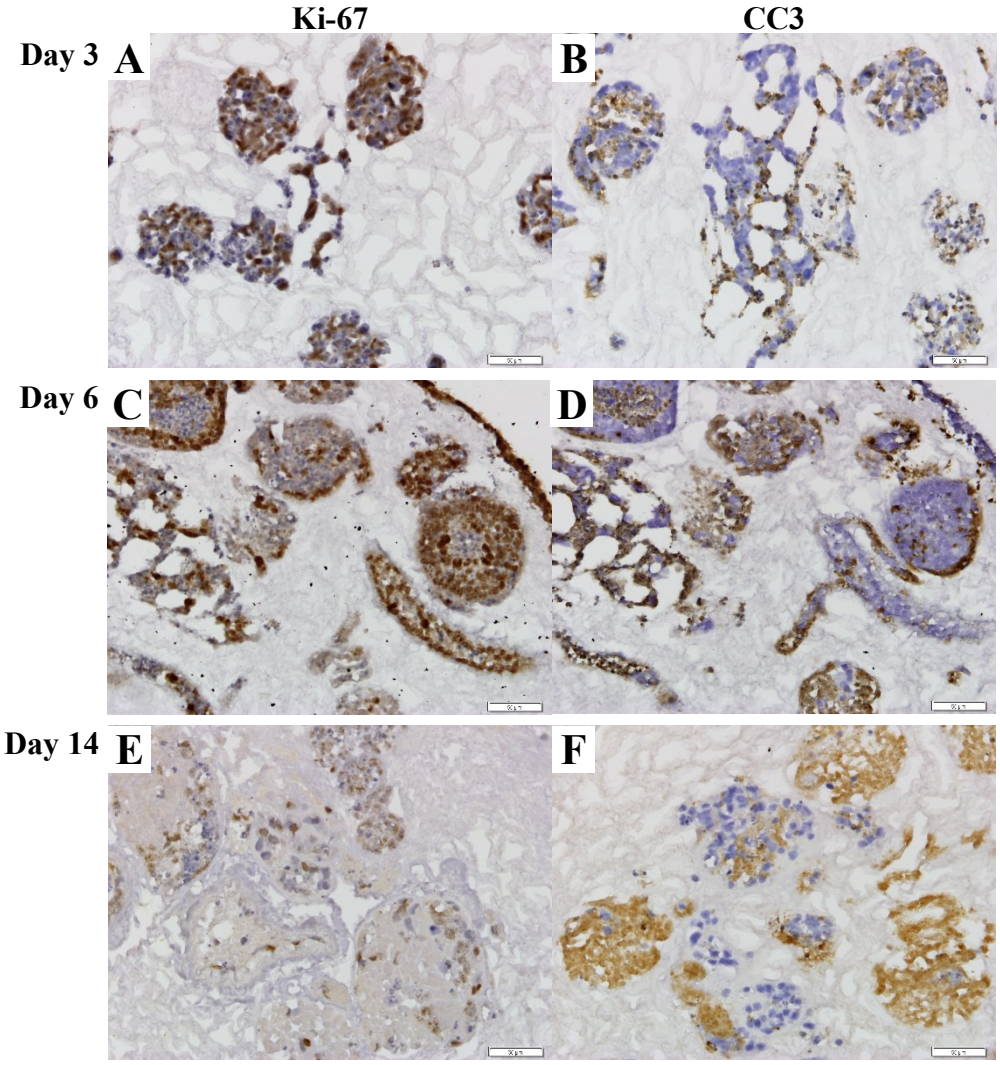

Figure 4-6. Patterns of ES cell division and apoptosis on section cultured scaffolds reflected with KI-67 and Cleaved Caspase-3 (CC3) IHC (40x magnification, bars denote 50μm). Scaffolds decellularized using *Kidney SDS* protocol. A, B) At day 3, cell division is strong in glomeruli and interstitium (left), yet there is also significant apoptotic signal arising in those areas (right). C, D) By day 6, serial sections show cells adjacent to vessel walls remain strong in proliferation, as well as cells on the capsule and in scattered locations including interstitium and filled tubule cavities (left). In contrast, there is pronounced signal for apoptotic residue in glomeruli, and some positivity in smaller vessels, interior of larger cell-filled areas, and interstitium (right). E, F) Over longer culture times, 14 days, scattered cells are still Ki-67 positive (left), and a diverse landscape of CC3 demonstrates a dynamic history of cell turnover (right). At these culture times, many intact cells do not show positivity for CC3 or Ki-67, indicating a degree of stability.

renal differentiation may be targeted. The following analyses were performed on scaffolds produced from the *Kidney SDS* decellularization protocol. Initially, the cultured scaffolds were

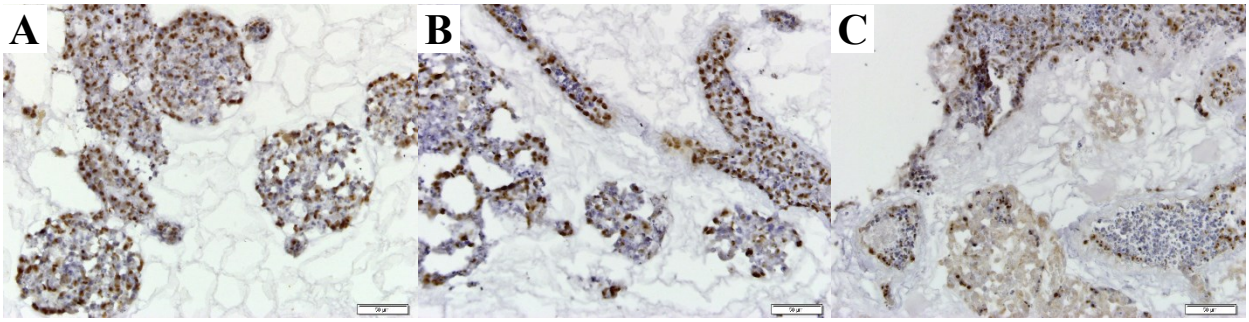

Figure 4-7. Retention of transcription factor Oct-4, regulator of pluripotency, in B5/EGFP ES cells section cultured on acellular kidney scaffolds produced with the *Kidney SDS* protocol (40x magnification, bars denote 50μm). A) Most cells are positive at day 4. B) At day 6, a significant number of cells retain positivity in the glomeruli, interstitium, and vessels. C) At day 14 of culture, scattered groups of cells remain positive.

examined using Oct-4 immunohistochemistry (Figure 4-7). Oct-4 is a key factor in regulation of ES cell pluripotency. It has been used as a marker of undifferentiated stem cells with loss of Oct-4 signifying differentiation.[34, 122, 179] Oct-4 is present in undifferentiated B5/EGFP mES cells. At day 4 of culture in the scaffold, Oct-4 is indiscriminately retained in approximately half of all cells. This is slightly diminished at day 6, with cells in the interstitium and vessel lining containing a greater portion of positive cells. At day 14, scattered groups throughout the scaffold are still Oct-4 positive.

Cytokeratins are intermediate filament proteins that commonly mark epithelial differentiation. They are present in many adult kidney cells including those in the proximal tubule, distal convoluted tubule, straight portion of distal tubule, and collecting duct.[153] They are extensively present in the developing metanephros yet are absent from undifferentiated metanephric mesenchyme.[206] Figure 4-8 shows cytokeratin positivity at day 10 of seeded scaffold culture. Cells were positive in groups throughout the scaffold, and readily identifiable in the glomeruli, Bowman's capsules, interstitium, and vasculature. Cells lining larger vessels, some assuming a squamous morphology, were positive. There may have been cytokeratin positivity in cells located in the tubules, but interpretation of location was imprecise due to

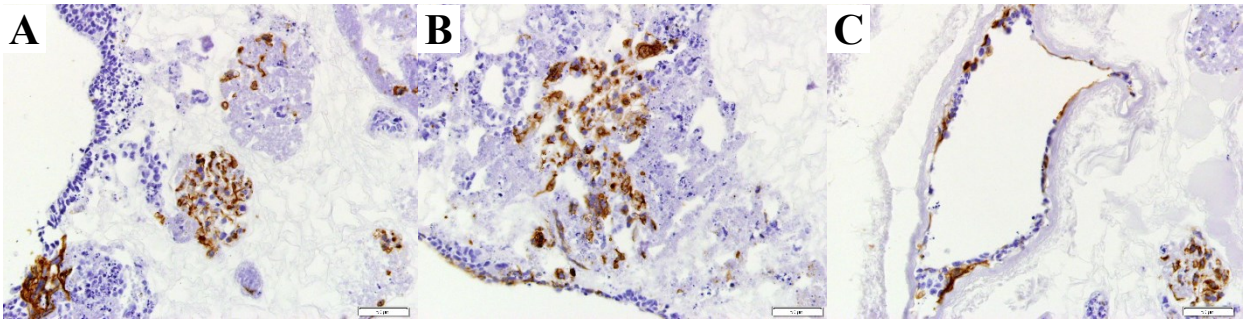

Figure 4-8. Pan-cytokeratin IHC reveals epithelial differentiation of seeded B5/EGFP ES cells at 10 days of sectioned scaffold culture (40x magnification, bars denote 50µm). Scaffolds were produced using the *Kidney SDS* decellularization protocol. Positive cells were identified in the A) glomeruli, B) interstitium, and C) lining vascular basement membranes.

scaffold deformation. Cytokeratins were not detected in undifferentiated embryoid bodies. Reference cytokeratin detection in an embryonic kidney is shown in the appendices, Figure B-1.

Given overall drop in Oct-4 expression and presence of epithelial differentiation, immunohistochemistry was performed on cultured scaffolds for three key proteins involved in kidney organogenesis, Pax-2, WT1, and Ksp-cadherin. Pax-2 identifies a handful of cell populations in the developing metanephros, including intermediate mesoderm, ureteric bud, and cap mesenchyme. It is a key regulator of branching morphogenesis and MET, and is necessary for differentiation of early stem cell populations such as cap mesenchyme and Foxd1+ stromal cells.[38, 58, 65, 68] WT1 is present in the metanephric mesenchyme and proximal renal vesicles during development.[60, 69, 70] WT1 promotes differentiation to renal vesicle cells by suppression of Pax-2.[70] Pax-2 and WT-1 proteins are both transcription factors located in the cell nucleus.[151, 152] Ksp-cadherin is a transmembrane cell adhesion protein found exclusively in the kidney.[119, 154, 155] It can found on the basolateral membranes of epithelial cells in the ureteric bud and related progeny. It is also present in non-glomerular epithelial cells of the nephron after polarization, which typically occurs during onset of active glomerular filtration.[155] Detection of these three proteins (Pax-2, WT1, and Ksp-cadherin) on mES cells cultured on decellularized kidney

scaffolds may indicate differentiation to renal lineages. After IHC was performed, Pax-2 was found present in diverse locations at all tested time points, although no evidence of WT1 was discovered. Ksp-cadherin was absent at any location at multiple time points except for one anomalous cluster of cells, which shall be considered inconsequential as it was not repeatable. None of these proteins were found present in undifferentiated B5/EGFP cells. Pax-2 expression persisted from day 3 to day 14 of culture (Figure 4-9). At day 3, a majority of the intact cells were positive in all populated areas including glomeruli, arterioles, and interstitium. At day 6, most of the intact cells remained positive, including cells now lining calyces, large vessels, and on the capsules. At later time points, 10 or 14 days, Pax-2 positivity was not as consistent in intact cells as previous time points; many cells were Pax-2 negative. However, immunopositive cells still remained at all mentioned locations with the addition of cells that were scattered within tubules and those that have populated the Bowman's capsular spaces. Interestingly, cells that have progressed into less compact groupings show Pax-2 positivity. One example is cells that have remained viable after most of the original glomerular seeded cells have become apoptotic. Reference IHC images for Pax-2 and Ksp-cadherin on normal adult and embryonic kidney tissue is available in Appendix B (Figure B-1).

Embryoid Body Culture

One method to initiate differentiation of ES cells and promote generation of simple tissue structures is to culture the cells as embryoid bodies (EBs), or suspended cell aggregates.[116, 117] Comparison of EB culture with seeded scaffold culture allows analysis of ES cell differentiation without the influence of kidney extracellular matrix composition and structure. Therefore, B5/EGFP ES cell EBs were generated and cultured using the hanging drop method.[207] Media composition was equivalent to that used in the cultured scaffold experiments. Once cultures were completed, immunohistochemistry was performed on these EBs; a summary of differentiation

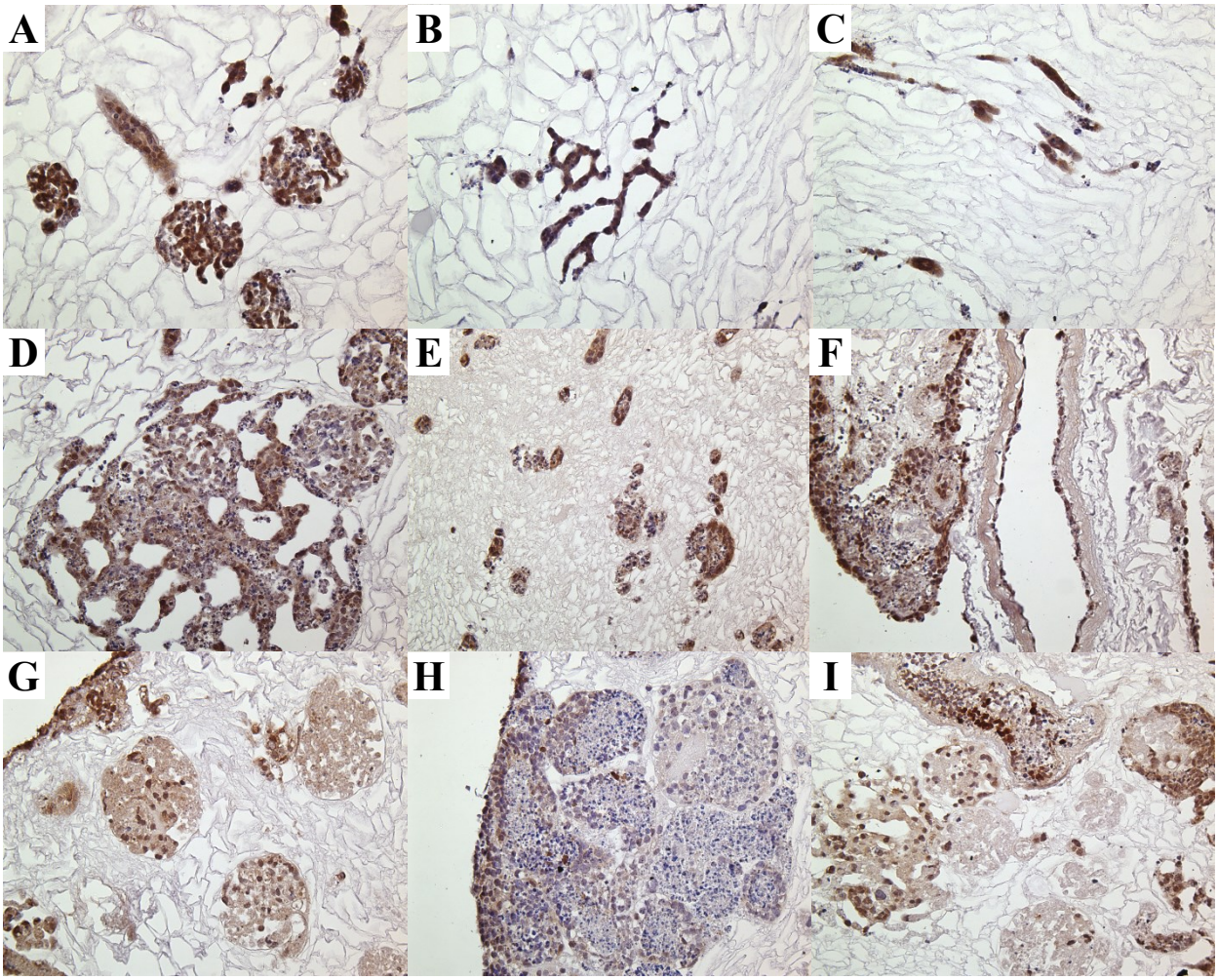

Figure 4-9. Immunohistochemical analysis of Pax-2 expression in ES cells section cultured on acellular kidney scaffolds produced using *Kidney SDS* protocol (40x magnification). Cells showed widespread positivity by day 3 at all locations including A) glomeruli and arterioles, B) interstitium, and C) inner medulla. At day 6, cells were immunopositive D) in the glomeruli and adjacent interstitium, E) in the inner medulla, and F) lining large vessels and on the capsule. G) At day 10, despite previous apoptosis, pax-2 positive cells remained in the glomeruli; H) cells were also found lightly scattered throughout packed tubules and lining the capsule. I) At 14 day of culture, pax-2 cells persisted in various locations, including interstitium, in glomeruli, in the Bowman's space, and in vessels.

markers in comparison with seeded scaffold culture is given in Table 4-1. Figure B-3 in the appendices displays relevant histological images. Embryoid bodies show a dynamic tapestry of cell division and apoptosis. The small densely aggregated ES cells with high nucleus-to-cytoplasm ratio progressed into alternate cell morphologies and less compact arrangements, with

Table 4-1. Immunohistochemical detection of embryoid body differentiation compared to seeded acellular kidney scaffolds.

Tissue	pan CK	WT1	Pax-2	Ksp-cadherin
EBs Day 0	-	-	-	-
EBs Day 10	+	ND	+	-
Kidney Scaffold Day 10	+	-	+	-

Embryonic stem cells seeded into scaffolds decellularized with the Kidney SDS protocol or cultured as embryoid bodies (EBs) are both positive for key proteins in renal organogenesis. Cytokeratins (pan CK) are extensively present in epithelial cells of the developing metanephros, but absent from undifferentiated mesenchyme. WT1 is a transcription factor present in metanephric mesenchyme and proximal renal vesicle of developing nephron. Pax-2 is a transcription factor present in cell populations of intermediate mesoderm, ureteric bud, and cap mesenchyme. Ksp-cadherin is present in ureteric bud epithelium and appears after non-glomerular epithelial cells in the nephrogenic zone have polarized.

Key: -, protein not detected; +, protein detected in multiple locations in proximity of implanted cells; ND, no data

each embryoid body transforming into unique heterogeneous cell collections. There was also a continuous loss of Oct-4 expression. As stated earlier, at day 0, there was no evidence of cytokeratins, Pax-2, WT1, or Ksp-cadherin positivity. At day 10 of culture, cytokeratins were present on the periphery of the bodies and adjacent to interior spaces. Pax-2 immunopositivity was visible in a significant quantity of cells. Ksp-cadherin was not detected. WT1 IHC was not available at this time point.

Discussion

Tissue Engineering Scaffold Cultured with Embryonic Stem Cells

In this research, kidneys have been decellularized to produce whole organ extracellular matrices. These matrices were designed to be used as tissue engineering scaffolds for the generation of implantable kidneys. Other groups have used whole organ ECM scaffolds from heart, liver, and lung to create functional constructs.[18, 22-24, 26] These types of scaffolds, if properly prepared, can promote cell attachment, proliferation, differentiation, and organization into tissue-specific structures.[20, 24] One particular challenge in kidney scaffold-based tissue

engineering, given the complex tubular architecture of renal ECM, is repopulation of the scaffold with the requisite epithelial cell types to reinstate filtration and reabsorption functionality. One potential solution, given that ECM components can influence differentiation of pluripotent stem cells, is to deliver these cells into the scaffold and allow them to selectively differentiate into the required somatic cell types in response to the underlying heterogeneous ECM.[20, 30, 160]

To test the response of murine ES cells to whole decellularized rat kidney, cells were delivered into the renal arteries and cultured up to 14 days. Initial distribution of cells and subsequent expansion patterns were observed with fluorescence microscopy. Hematoxylin and eosin histochemistry was then used to inspect cell localization and morphological changes in relation to the underlying basement membrane. Immunohistochemistry was employed to detect patterns in cell division, apoptosis, and differentiation. The antibodies used in the differentiation studies targeted proteins expressed in epithelial cells and early stage nephrogenic progenitor cell populations. Also examined was a pluripotent stem cell marker, the loss of which signified differentiation. To compare response of these ES cells to those uninfluenced by ECM, embryoid bodies were cultured to equivalent time points and differences in cell proliferation, apoptosis, and differentiation were noted. Also during this study a perfusion culture method was examined. Finally, to improve delivery of cells to the tubules and collecting ducts, ureteral seeding was explored.

Cell Distribution

In order to effectively repopulate a tissue scaffold, cells must either be delivered to all areas of the scaffold or be able to proliferate and migrate to fill voids. Once mES cells were injected into the renal artery of a rat kidney scaffold produced with the Kidney SDS decellularization protocol, a majority were captured in the glomerular capillary tufts. These seeded glomeruli were effectively distributed throughout the cortex and outer medulla of the

kidney. In a model of glomerulonephritis repair, delivery of MSCs through the renal artery also localized to the glomeruli, achieving a 70% distribution.[16, 83] Assuring that cells are delivered to the glomeruli is critical given the cell requirement for normal glomerular filtration. After delivery, the most probable scenario is that during the first 6-8 days in section culture, cells remained confined to an intact vascular system, progressing outward from the glomeruli into the efferent arteriole and peritubular capillary system (including vasa recta) or back into the afferent arteriole and larger arteries. The peritubular capillary system is located within the interstitium in both cortex and medulla; this could explain cell presence in both locations. At 6-8 days of culture, cells sporadically appeared in tubules, in the Bowman's capsule space, lining the calyces, and on the exterior kidney capsule. It may be, however, that appearance of cells in these alternate locations is due in part to the section culture method; cells could have migrated outside the boundary of the section and returned in an adjacent location. In whole kidney static culture, cell appearance outside of vascular locations was markedly reduced, with the caveat that those culture samples were produced with different decellularization protocols; those protocols may have had a different effect on ECM composition and condition, which effects the ability of the cells to migrate.

Delivery of cells through the renal artery of the kidney scaffold was an effective means of dispersing cells. However, a robust vascular basement membrane may be inhibiting the ES cells from migrating to other areas of the kidney, such as the tubules or collecting ducts. Based on smooth muscle cell infiltration of arterial matrix, it has been suggested that migration is dependent on density of the matrix.[101, 103] Other cells have been successful in passing through the vasculature. Uygun *et al*. (2010) used the vascular supply to repopulate an SDS-produced whole liver scaffold with primary hepatocytes.[26] In a model of tubulonecrosis repair, an injection of

adult renal stem cells successfully passed through the vessels and localized to damaged tubules.[76] However, this movement may have been motivated by chemoattractants released by the injured cells.

Another potential route for delivery of cells into the kidney scaffold is through the intact ureter. Injected cells have the potential to reach past collecting ducts and tubules all the way to the epithelial side of the glomeruli in the Bowman's capsules. When ES cells were injected into the ureters of the kidney scaffolds, cells did reach the glomeruli but for the most part were unexpectedly localized to the interstitium (Figure 4-5). In addition, this delivery method was ineffective and distributing cells through the cortex and medulla; only sparse radial streaks or wedge shaped sections were observed. A majority of the cells were retained in the calyceal spaces or passed through the scaffold altogether. It may be that delivery pressure of the cell solution exceeded mechanical tolerances of the basement membranes causing ruptures and emptying or rerouting of cells, or that the anatomy of the rat kidneys is not entirely conducive to retrograde cell delivery. On a positive note, many cells did arrive in the tubules and over 10 days of culture filled those spaces. Despite its potential, this route of cell delivery was not pursed because of low reliability and inconsistency in cell placement. In addition, scaffold culture permitted cells delivered into the renal artery to move into tubules over time, allowing assessment of those areas. In the future, however, revisiting and improving ureteral cell delivery would be beneficial, as a culture method that retains the whole kidney scaffold is necessary to achieve a functioning organ. One method of improving cell seeding in whole organs is to divide delivery of a single cell suspension into multiple injections and space them at an even time interval.[26] If ureteral delivery is refractory to improvements, another option is direct microinjection of cells into the body of the kidney.[80, 130]

Precise identification of cell location in the scaffolds was challenging and open to interpretation, especially in the Kidney SDS scaffolds, as all of the cell features typically used for labeling in normal kidney histology had been washed away. In this research, interstitial spaces were identified as the narrow basement membrane spaces surrounding the larger spaces. The larger spaces were likely proximal or distal tubules or collecting ducts and housed the bulk of the epithelial cell population. In early iterations of the kidney decellularization protocol, cellular debris was located within these larger spaces, supporting this assertion. The primary caveat to using ECM spacing to identify interstitium is that at later time points in Kidney SDS scaffold cultures, severe scaffold distortion made this method impractical. In the future, precise labeling of ECM could be achieved with IHC, such as with combinations of individual laminins or collagen chains.[10]

Cell Proliferation

An effective tissue scaffold promotes viability of implanted cells and supports proliferation to assure ECM coverage. Other factors play a role in this such as growth media constituents and overall cell culture environment. To evaluate the Kidney SDS scaffold, an IHC study was performed to assess any tendencies toward seeded ES cell proliferation or apoptosis. It was immediately clear that ES cells maintained a high level of cell proliferation and apoptosis in all areas of the kidney. Significant variability made attempt to correlate these events with adjacent extracellular matrix challenging, yet a few trends did present themselves. After 3-6 days of culture, a bulk of the cells in the glomeruli strongly expressed the protein marker for apoptosis, CC3. This may be due to crowding and a lack of nutrient/waste diffusion at that location or a lack of sufficient intact basement membrane proteins in the glomerular capillary to inhibit ES cells apoptosis. It may also be a result of overall limited cell survivability in the scaffold under the culture conditions; many of the glomeruli were initially seeded and would

likely be the first to become apoptotic. Cells lining the calyces, capsule, and open vascular basement membrane had a tendency to resist apoptosis and express the marker for cell division, KI-67. In contrast, cells on the interior of crowded vessels, or on the interior of large ES cell aggregates have shown increased CC3 protein expression (Figure 4-6). Embryonic stem cells are, by nature, very dynamic. Upon observation of standard EB development, ES cells that are adjacent to ECM proteins will proliferate while unattached cells become apoptotic.[208] This is a basic part of intended cavity formation and tissue structuring.[207] In other research, fetal kidney cells were allowed to migrate into decellularized kidney ECM, but both the cell explant and scaffold-cultured cells showed evidence of apoptosis after 5 days.[114] It may be that the duration of cell survivability in this research is comparable to other contemporary cell-seeding protocols.

During the first 4-6 days of cell culture on Kidney SDS scaffolds, seeded ES cells proliferated to the limits of the ECM boundaries and in most instances distorted the matrix. This matrix distortion is exacerbated by days 10-14, often resulting in a dramatic reduction in total graft volume. Cells cultured on scaffolds that retained cell debris, such as produced with the Kidney DOC and Graduated 10% protocols filled the ECM spaces without distortion. This may be due to residual cellular material stabilizing the matrix. Another consideration is that SDS may be causing structural changes to the ECM, as the protocols that left residual cell debris utilized NaDC. Cortiella *et al*. (2010) saw scaffold shrinkage after implanting ES cells into an acellular lung.[20] The lung was decellularized using SDS. Nakayama *et al*. (2010) saw acellular kidney shrinkage after use of SDS, but before cell seeding.[114] Those scaffolds, however, were produced at an elevated temperature of 37°C.

Cell Differentiation

Fundamental to the use of ES cells to repopulate an acellular kidney scaffold is differentiation based on ECM contact. Extracellular matrix has been shown to regulate gene

expression, cell phenotype, and promote differentiation of ES cells.[20, 30, 160] In addition, 3D matrices have been shown to improve differentiation of ES cells through increased cell-cell and cell-matrix association.[209] The purpose of the scaffold culture experiments conducted herein was to establish a baseline cell response without post-decellularization scaffold modification or addition of culture media factors that promote renal-specific differentiation. Therefore, as an initial histological assessment, mES cells injected into Kidney SDS ECM scaffolds were cultured and observed for morphological changes. Initially, the cells appeared dense and crowded with a high nuclear:cytoplasmic ratio, and many of these cells seemed to remain as such throughout the duration of the 14 day culture. After day 6, some cells adjacent to vascular or tubular basement membranes assumed a more cuboidal morphology. Alternatively after that time point, cells that were uncrowded and attached to the ECM of larger open spaces such as arterioles, arteries, and calyces displayed a squamous morphology. Some cells in the glomeruli and in interstitium at days 10 and 14 of culture assumed a more relaxed spacing; interestingly, this appeared to correlate to areas with high levels of apoptotic cell residue. One possibility is that if the apoptotic cells were confined to the vasculature, then these cells were located outside the capillaries such as on the epithelial side of the GBMs or exterior to the peritubular capillaries yet still contained in the interstitium.

In an attempt to elucidate the observed morphological evidence of differentiation, IHC was conducted. Oct-4 is a marker of undifferentiated ES cells, and loss of expression has commonly signified differentiation.[122] Over the course of 14 days of culture, Oct-4 expression, although diminished over time, still had significant level of expression in non-apoptotic cells (Figure 4-7). However, cytokeratin IHC clearly showed epithelialization of cells in groups throughout the scaffold, including in the glomeruli, on the capsule, in the interstitium, and lining

the large vessels (Figure 4-8). The next three antibodies detected proteins that are expressed in early nephrogenesis, Pax-2, WT1, and Ksp-cadherin. Combinations of these three proteins are expressed in all early kidney progenitor cell populations. They are also present in adult kidney cells, but the ES cells are likely to pass through a progenitor population before progressing to terminally differentiated adult kidney cells. According to gene expression, differentiation of ES cells in embryoid bodies typically follow traditional developmental pathways.[122] Pax-2 was present in a majority of the seeded mES cells throughout the duration of the culture (Figure 4-9). Pax-2 is present in late-stage intermediate mesoderm, ureteric bud and epithelial progeny, induced metanephric mesenchyme, and the cap mesenchyme stem cell population.[59, 60, 121] It is commonly used to identify kidney-specific progenitor cell populations.[34, 114, 119, 122] WT1 is present in metanephric mesenchyme and cap mesenchyme and Ksp-cadherin is present in ureteric bud and related progeny.[60, 69, 70, 119, 154, 155, 210] Therefore, the only remaining possibility for Pax-2 positive cells is late-stage intermediate mesoderm. This does not guarantee renal specific lineage however, as Pax-2 has also been detected in development of mid-hindbrain, optic nerve, inner ear, and female genital organs.[67, 211] Proving that the Pax-2 positive cells belong to the intermediate mesoderm cell population would require detected expression of other proteins such as Osr1, Lhx1, and Emx1. [60, 63]Pax-2 is also present in later stages of kidney development, such as in pretubular aggregates derived from cap mesenchyme or adult proximal tubules cells, but these cells would also be positive for WT-1 or Ksp-cadherin.[119] Cytokeratin IHC was not performed in conjunction with the Pax-2 IHC, so co-localization could not be used to further isolate the Pax-2 cell population. In addition, the cytokeratin IHC employed detected a broad spectrum of cytokeratins and therefore could not be used to mark specific kidney epithelial cells. It may be that the cytokeratin expressing cells and Pax-2 expressing cells are separate cell

populations. Also, with absence of Ksp-cadherin and WT1, cytokeratin positive epithelial cells are not specific to the kidney. Finally as Pax-2 and cytokeratin expression was not limited to specific basement membranes types, preferential differentiation could not be recognized.

In the future, it may be beneficial to complement IHC with qRT-PCR (quantitative reverse transcriptase polymer chain reaction) analysis for detection of gene expression markers. Immunohistochemistry analysis is based on localized protein concentration and visual interpretation of the observer, while qRT-PCR quantifies total mRNA in a sample.[212] Given the heterogeneity of the kidney ECM, however, a method for targeting specific areas in the tissue sample such as laser microdissection may be required for location-based comparison.[119] Use of qRT-PCR may support IHC results or offer alternative data for cell identification. The primary caution in comparing the two results is that there are numerous opportunities for variability during processing of both techniques and total protein may not correlate with total mRNA.[212]

The markers selected for identification of early renal progenitors, Pax-2, WT1, and Ksp-cadherin are not unique to any particular nephrogenic cell population. Additional markers would serve to clarify differentiated phenotypes. One valuable resource in the selection of these markers is a collaborative database of genitourinary development, GUDMAP. It contains known gene expression profiles and matched IHC for every identified nephrogenic cell population. It also identifies anchor genes, which are unique to a particular cell population.[196, 197]

Embryoid Body Comparison

One method to promote ES cell differentiation and tissue formation is to allow the cells to form three dimensional aggregates called embryoid bodies. Embryoid bodies develop into heterogeneous structures and cell differentiation paths may be effected by media, culture environment, size of EBs, and composition of adjacent ECM.[207] In this research, EBs were formed from the mES cells and cultured up to 14 days in the same media used for scaffold

culture. In order to examine the effects of kidney ECM, EBs were compared to the mES cells cultured in the scaffold. CC3 and KI-67 immunohistochemistry showed interior cells undergoing apoptosis with a majority of the remaining cells undergoing cell division (Figure B-1). This behavior is typical of EB development and cavity formation; cells near the exterior produce ECM, notably collagen IV and laminin, which in turn promotes the survival of those cells.[207, 208] Similar behavior was observed in scaffold culture whereby cells attached to vascular basement membrane had a tendency to express KI-67 while unattached cells in the lumen expressed the apoptotic marker CC3.

At day 0 of culture, none of the EBs showed immunopositivity for the markers of differentiation pan CK, WT1, Pax-2,or Ksp-cadherin (Table 4-1). After 10 days of culture, both the EBs and cells in the scaffold showed positivity for pan CK and Pax-2 and absence of Ksp-cadherin. In the EBs, Pax-2 was expressed in most cells with CKs present on exterior borders and developing interior spaces. This distribution of proteins is similar to cells cultured in the scaffold. There is no data for WT-1 beyond day 0. In other EB differentiation research, mES cell lines EB3, D3, R1, and 129-sv-ES all showed Pax-2 mRNA expression by day 6 of culture, Ksp-cadherin by day 7, and WT1 by day7.[119, 122, 195] However, there was variation in the expression profiles. For example, qRT-PCR analysis of the EB3 culture showed initial KSP expression at day 7 with sharp increases at day 12 and 18, whereas a densitometric RT-PCR analysis of the D3 culture showed Ksp-cadherin presence at day 1 with gradual increase to day 14.[122, 195] Both of these results contrast with the absence of Ksp-cadherin in this research. However, comparison between all these results may not be reasonable as the cell lines, media conditions, and detection methods were all different. On the other hand, there may be a case for additional analysis of both the cultured scaffolds and EBs with an mRNA detection method such as qRT-PCR.

Given the limited IHC comparison of B5/EGFP mES cells cultured in acellular kidney scaffolds or as EBs, there is insofar no evidence that the Kidney SDS scaffold promoted differentiation of the ES cells beyond inherent tendency. However, as described, the analysis is limited, and further examination may reveal evidence of improved differentiation. Traditional IHC is exceptional at pairing protein expression with tissue location, but it is not a quantitative analysis. QRT-PCR or ELISA could be employed to quantify any differences in mRNA or protein expression, with tissue segments isolated by microdissection. One consideration is that the timing of gene expression in the seeded cells may lag behind EBs, as the seeded cells need time to expand into the matrix and respond to extracellular signals. A better comparison may be to examine the response of mES cells cultured in two different types of acellular matrices. Another consideration is that ES cells cultured in the Kidney SDS scaffolds showed strong tendency for proliferation, often crowding into spaces. This may affect overall trends in differentiation as a reduced number of cells are in contact with the ECM.

Endothelial Differentiation

Based on histological observation many of the injected mES cells were assumed to be retained in the kidney scaffold vasculature. Therefore an opportunity existed to test the cells for endothelial differentiation driven by contact with intact vascular basement membrane. Analysis of the cultured scaffold samples was performed at the lab of Dale Abrahamson at the University of Kansas Medical Center. Using IHC, they found that the cultured cells were positive for both Bandeiraea simplicifolia lectin B_4 (BsLB4) and VEGFR2 (Flk-1).[213] BsLB4 lectin is a highly selective carbohydrate binding protein that marks cells of endothelial lineage, including mesoderm-derived angioblasts.[214] VEGFR2 is the receptor for VEGF growth factor and signaling between the two is necessary for vascular development.[215] In other work, it was suggested that one source of endothelial cells in the kidney are Flk-1+ cells in the metanephric mesenchyme.[62]

However, lack of WT-1 protein detection in this research implied the mES cells cultured in the kidney scaffold did not transition through a metanephric mesenchyme phenotype. An informative experiment would be to analyze the cultured scaffold for any VEGF retained in the decellularized kidney. One group decellularized whole liver using Triton X-100 and SDS and found VEGF (which can bind to ECM) present in the scaffold.[21] It was shown that VEGF can promote human ES cells into functional endothelial cells.[216] It was also shown that the application of shear stress will induce differentiation of Flk-1+ ES cells into vascular endothelial cells *in vitro*.[202]

Perfusion Culture

A system was designed to perfuse media through the cell-seeded kidney scaffold in a pulsatile manner approximating rat cardiac output. Vascular perfusion of media improves oxygen and nutrient delivery to cells, removes products of cellular metabolism, and allows thicker tissue growth.[198] In addition, as fluid flow plays a role in regulation of renal cell epithelium, perfusion of media allows closer simulation of the *in vivo* cellular environment.[200, 201] Simulation of *in vivo* microenvironment improves tissue-specific cell growth, differentiation, and structure formation.[22-24, 201] Other groups have used perfusion bioreactors to grow lung, heart, and liver tissue.[22-24, 26]

In this research, mES cells were seeded into acellular kidney scaffolds and given 12 hours to adhere before perfusion of media was initiated. As in static culture experiments, the initial injection contained $2x10^6$ cells. However, by day 3, there was a significant reduction in cells as compared to static scaffold cultures. The number of cells retained in all scaffolds after a seed injection was fairly consistent, so one possibility for the cell reduction is that once flow commenced non-adherent cells were dislodged and passed out of the kidney. At day 6 of culture, renal corpuscles were observed with cells packing the Bowman's space, yet none in the

associated glomerular capillaries. An explanation for this is that stress from media circulation caused a rupture in the GBM, allowing cells to pass into the tubules. This may also account for the increased tubule seeding associated with perfused culture. The other possibility for overall cell reduction is that media conditions or shear stress reduced proliferation of the ES cells. The cells as observed at day 3 of culture were uncharacteristically isolated so reduction of proliferation may at least be a contributing factor.

All scaffolds used in perfused culture experiments were produced from the *Kidney NaDC* protocol, and as a consequence, retained cellular debris. This debris would have affected media flow patterns and stress distribution, possibly influencing condition of the cells or contributing to ECM rupture. This is compounded by the fact that a majority of cells were delivered to the glomerular capillaries, and since they were not flattened endothelial cells, served to block fluid flow. Conducting future experiments with a debris free Kidney SDS scaffold would be a worthwhile experiment. Further, once the technique for ureteral seeding is improved, shifting the cell injection to a ureteral seed would allow unimpeded media flow through the glomeruli. This may enhance growth and epithelial differentiation of cells in tubules and collecting ducts.

One reason the perfusion culture experiments were not pursued for the duration of this research study was because of the overall reduction in cells compared to static culture methods. Two other reasons were comparative cost of the experiments and lack of ability to compare one cell seed/scaffold combination over multiple time points, which was accomplished with section scaffold culture. Therefore, analyses of cell proliferation, apoptosis, and differentiation were not conducted. Despite the overall reduction in cells, there may have been positive shifts in cell response such as increased expression of endothelial or epithelial markers. Yamamoto *et al*. (2005) showed that fluid shear stress promoted differentiation of Flk-1 positive ES cells to

vascular endothelial cells *in vitro*.[202] The Flk-1+ ES cells were induced by culture on collagen IV.

Despite the reasons the perfusion culture experiments were temporarily sidelined, use of a bioreactor that mimics *in vivo* circulation, as shown in other studies, remains the most promising system to culture cells in a kidney scaffold. In addition, while section scaffold culture is effective for analysis of cell response in the short term, given the disruption of the whole intact matrix, it is not a method that will lead to an implantable kidney construct. In order to continue with perfusion culture, two design flaws that may lead to an adverse cell response need to be addressed. Pressure-driven gas flow was designed to improve diffusion in the media, however, it was completely dehumidified. The media reservoir was significant (500ml) but the gas may have pulled enough moisture from the media to adversely change the osmolality of the culture medium. Also, the regulation of CO_2, and therefore culture pH, was challenging. It was discovered that the media tubing was highly permeable to gas, especially CO_2. Since a significant portion of the tubing passed outside of the incubator, there was opportunity for appreciable CO_2 loss. To compensate in the short term, CO_2 concentration in the incubator was increased to drive diffusion of the gas back into the media. Solutions to these problems are replacement of the tubing with low gas permeability and the addition of a cylinder washer for gas humidification.[22] Other potential improvements to this initial design include use of a membrane oxygenator for improved gas diffusion, use of a heat exchanger instead of an incubator to control temperature, and replacement of the vertical pressure regulation circuit with a sealed, pressurized chamber.[22, 183]

Culture Media Modifications

One goal of this research was to assess the ability of the decellularized kidney scaffolds to promote renal differentiation of mES cells using a basic growth media without the influence of

additional growth factors or other pro-differentiation media additives. In future work, however, modification of the base media or addition of these factors could be used to enhance the innate potential of the scaffold to control the fate of seeded cells or even promote the cells to a fully differentiated phenotype. In the base media, each ingredient has potential to affect cell fate. For example, the antimycotic added to the differentiation media in this research, Fungizone® (Amphotericin B), had potential to effect cellular gene expression and modify results.[217] One potential avenue for improved differentiation is substitution of the base media with other formulations that have been used to differentiate ES cells into renal lineage. Morizane *et al*. (2009) used DMEM, 10% FBS, and 0.1 mM 2-mercaptoethanol while testing specific growth factors in their studies of differentiating both ES cells and IPSCs into renal lineages.[122] The Morizane *et al*. study also used less FBS than used in this research.[122] Fetal bovine serum, a primary ingredient in most growth media recipes, contains a high content of embryonic growth factors and other components that have been shown to support cell metabolism. It is commonly used in ES cell media to promote differentiation. The drawback to FBS use in media is that not all components are identified and batch to batch variability.[31] This variability can influence potential to differentiate cells along a certain lineage. To find improved base growth media formulations it may be worth trying different batches of FBS, or reducing FBS requirement altogether. Other potential sources for base media recipes are those used for metanephric organ culture. These formulations are designed to maintain all cells in a developing kidney. Nakayama *et al*. (2010) used DMEM/F12, 25 ng/mL prostaglandin E, 1% insulin/transferrin/selenium, 10% FBS, and 1% penicillin/streptomycin to support growth of fetal kidney cells from whole explanted metanephros into adjacent decellularized kidney matrix.[114] Giuliani *et al*. (2008) was able to extend average metanephric culture time to 10 days before necrosis using Leibovitz's L-

15 base medium, 1% penicillin/streptomycin, and 2% FBS. Reduction of FBS to 2% in this case actually reduced necrosis over span of the culture period while supporting normal nephrogenic development.[218]

In addition to base media changes, other additives such as growth factors may be used to promote renal differentiation of the mES cells. These may be applied to ES cultures before injection to pre-differentiate cells and allow use of marker-specific cell sorting for population purification, after injection to allow differentiation enhancement by the ECM, or both.[219] Promising targets for factor-mediated mES cell differentiation are one of the kidney progenitor populations. These include intermediate mesoderm, the precursor to all cells in the kidney; ureteric bud epithelium, the precursor to all collecting duct cells; or metanephric mesenchyme, the precursor to all cells in the nephron including vascular and stromal cells.[62] Morizane *et al*. (2009) claims 4 days of 10 ng/ml Activin exposure induces mesoderm differentiation, as shown by Brachyury expression.[122] Two other protocols use 10 ng/ml Activin and 0.1 μM retinoic acid for 5 days of culture to advance cells to an intermediate mesoderm state, although one of the protocols adds 50 ng/ml of BMP7 to reduce expression of the neuronal marker Synapsin-1.[121, 123] Morizane *et al*. also showed that once mesoderm was induced with Activin, that metanephric mesenchyme could be achieved per increased WT1 and Pax-2 expression with exposure to 150 ng/ml GDNF or 15 ng/ml BMP7 for an additional 10 days.[122] These markers may also indicate a more differentiated population such as cap mesenchyme or pretubular aggregates. Alternatively, continued exposure of Activin out to 14 days enhances Pax-2 and Ksp-cadherin expression, possibly indicative of ureteric bud, collecting duct epithelium, or tubular epithelium.[122] Other studies suggest hepatocyte growth factor (HGF) may promote tubular differentiation.[36, 179] Wnt-4 plays a role in aggregation and polarization of epithelia, and may be another useful culture

additive.[179] In regard to the kidney scaffold culture medium, it would be useful to test any of the above growth factor programs. The addition of these factors could encourage kidney-specific structure formation in an ECM scaffold. A simple first pass would be addition of 10 ng/ml Activin to the growth medium for the duration of the culture period to see if it induces Ksp-cadherin expression.

Conclusion

The first important step after creation of a tissue scaffold is to characterize the response of implanted cells. Once this is achieved, scaffold preparation and growth conditions can be modified to enhance functional tissue formation. In this research mES cells were seeded into decellularized whole kidneys and cultured up to 14 days. Cell delivered through the renal artery localized to the glomeruli and adjacent vasculature, but in section culture were to migrate to the tubules, capsule, and calyces over time. These cells were widely dispersed in the organ. Cells delivered into the ureter showed increased tubule or collecting duct localization, but dispersal through the organ was poor. Cells lining the arteries, in the calyces and on the capsule tended to express the marker for cell division. Cells delivered into the glomeruli or unattached to ECM tended to show increased apoptosis. Pockets of cytokeratin positivity indicated epithelial differentiation. Also widespread were markers of endothelial lineage commitment, VEGFR2 and BsLB4. Most cells expressed Pax-2, but there was an absence of WT1 and Ksp-cadherin, suggesting possible mesoderm differentiation. Limited IHC analysis of EB culture without kidney ECM contact also showed expression of Pax-2 without Ksp-cadherin expression, suggesting the kidney ECM scaffold may not have had a strong influence on renal-specific differentiation. Scaffold culture with perfused media resulted in reduction of cells, but an analysis of differentiation was not performed. Once ureteral seeding has been achieved, re-engaging the perfusion culture experiments with a debris free *Kidney SDS* scaffold and the

addition of renal inducing growth factors may be a promising step toward generation of a functional kidney.

CHAPTER 5
CONCLUSION AND FUTURE STUDIES

Research Summary

Extracellular matrix tissue scaffolds were created by the decellularization of rat kidneys. The decellularization process involved arterial perfusion of detergent-based solutions. The protocol utilizing SDS successfully removed cellular proteins and DNA while preserving ECM proteins collagen IV and laminin. Extracellular structures including a complete vascular network remained intact. In order to test the ability of the scaffold to support cell growth, mouse ES cells were injected into the renal artery. Embryonic stem cells have the potential to differentiate into all cells of the adult kidney. Proliferation and differentiation of these cells in response to the ECM would serve as a valuable step toward the generation of a functional kidney. Once injected, the cells primarily localized to glomerular tufts and adjacent capillaries. Unless the ECM was disrupted, a majority of these cells seemed to remain in the vasculature during culture. Contact with ECM promoted cell division and lack of contact promoted apoptosis. Immunohistochemical studies showed patches of epithelialization throughout the scaffolds. Of the three markers of kidney progenitor cell populations, only Pax-2 was detected. Ksp-cadherin and WT1 were absent from the cultured cells. Markers of endothelial lineage VEGFR2 and BsLB4 were both detected.

Through this research, a whole organ kidney scaffold was created and the response of seeded mES cells to the scaffold was examined. Given the specific decellularization protocols, cells, seeding method, and scaffold culture conditions, long term cell growth into renal structures was unlikely and kidney specific differentiation was limited. However, this research revealed multiple avenues not only for improving this outcome but for progressing toward a functioning implantable kidney.

Promoting Differentiation

There are two primary approaches for improving renal differentiation of the mES cultured in the kidney scaffolds: modification of scaffold production and addition of soluble factors to the growth media. The decellularization process involves a tradeoff between cellular material removal and preservation of basement membrane components. The best protocol for cell debris removal developed in this research was *Kidney SDS*. However, there is opportunity to reduce exposure to the decellularization chemicals while maintaining cell debris removal. This should improve retention of ECM embedded growth factors and growth factor associated proteins, ultimately leading to stronger influence over seeded cells. Extracellular matrix associated growth factors that play a role in kidney development include VEGF, PDGF, HGF, FGF, TGF-β, BMP-7, and Wnt-4.[52, 54-56] A prevalent kidney ECM protein that sequesters growth factors is heparan sulfate proteoglycan.[56] Once preserved growth factors are identified, a program of complementing growth factors or other additives to stimulate differentiation may be added to the scaffold growth media. If HSPG or other growth factor associated ECM proteins are preserved, loading growth factors into the scaffold before cell injection is an option.[171] One consideration in planning scaffold modifications or growth media additives is that ECM protein composition shifts during development.[10, 52] Another is that growth factor distribution and dose in an adult matrix is likely related to homeostasis of adult renal function and not entirely suitable to differentiate embryonic cells. A third consideration is that mouse cells are implanted in a rat matrix, so embedded rat growth factors may have reduced potency to effect mouse cell differentiation. These considerations may be partially addressed by supplementing the scaffold or media with mouse growth factors and increasing dose of growth factors shown to promote nephrogenic lineages, such as Activin, GDNF, and BMP7.[122] Qiao *et al*. (1999) showed that

soluble cytokines are all that is necessary to initiate nephrogenesis from renal progenitor populations.[86]

Scaffold Culture

It was estimated that most of the mES cells that were injected into the renal artery of the decellularized kidney scaffolds remained confined to the vasculature, but were able to eventually migrate into other areas due matrix disruption caused by the section scaffold culture method. In order to create whole organ tissue, however, the integrity of the scaffold must be preserved. Therefore, in order to access the tubules and collecting ducts, methods for improved ureteral seeding must be explored. These may include changes in injection pressure, solution volume, and total number of injections. It may also be that rat kidney is resistant to retrograde perfusion and another animal model must be used. One advantage to retrograde seeding of ES cells is that the cells are exposed to tubular and collecting duct ECM and associated growth factors from the onset, whereas the cells delivered into the vasculature may have been induced toward a vascular lineage and less responsive to other ECM after migration. Another advantage to ureteral seeding is that the vascular system may be populated with endothelial cells instead of reliance on endothelial differentiation of ES cells. Endothelialization of decellularized vasculature protects it from thrombosis, allowing the repopulated kidney to be implanted.[18, 23, 101, 103, 147, 199] Other cell-seeded scaffolds, such as from liver, heart, and lung have been endothelialized and implanted and have shown basic organ function.[18, 22-24, 26] Sources of endothelial cells include human umbilical vein endothelial cells (HUVECs) and autologous harvest, such as from saphenous artery or vein.[18, 22, 181, 199] Endothelial progenitor cells (EPCs), which can be isolated from peripheral blood, may also be used to line the vasculature.[181, 220]

The culture perfusion system designed in this research caused a reduction of mES cells in the scaffold, especially the glomeruli. This may have been caused from excessive pressure

disrupting the scaffold and allowing the cells to escape. This may also have been caused from fluid-induced shear stress reducing cell division. In any case, the cells localized to the tubules or collecting ducts did thrive. Therefore, once improved ureteral seeding of mES cells has been achieved, perfusion culture through the vasculature may be a valuable next step. Perfusion bioreactors have been shown in other seeded whole organ scaffolds to improve cell growth and epithelial differentiation.[22-24, 201] In regard to vascular endothelialization, perfusion culture also improves cell attachment, alignment, elongation, and overall patency rate.[24, 199]

Cell Alternatives

Once the scaffold and culture conditions have been optimized for renal differentiation of mES cells, testing the response of other cell sources may be worthwhile. Various adult kidney stem cells have shown ability to integrate into developing kidneys, but unless such cell lines were developed to be immune-tolerant, they would require biopsy for extraction and growth *in vitro* for subsequent autologous transplantation. In addition, it is doubtful that cells extracted from a diseased or damaged kidney would be suitable for new tissue growth. Multipotent stromal cells could be isolated from autologous bone marrow or donor-matched from a tissue bank. Although they have primarily demonstrated a supporting role in kidney repair, Yokoo *et al*. (2005) has shown that MSCs contribute to epithelial and interstitial cells when implanted into embryonic kidneys.[112] Amniotic fluid stem cells are another cell source that could take advantage of tissue banking. They are pluripotent and capable of renal-specific differentiation and structure formation.[32, 130] The most promising alternate cell to test, however, is iPSCs. They are a pluripotent cell source typically reprogrammed from adult autologous cells by forced expression of genes associated with embryonic cells.[33] Induced pluripotent stem cells have been differentiated into renal lineages and respond to the same inductive factors that influence ES cells.[122] Also, they have been reprogrammed from cell sources that do not require biopsy such as

mononuclear cells isolated from peripheral blood or tubule epithelial cells excreted in urine.[43, 44] Considerations for using those cells however are that peripheral blood cells are susceptible to age and mutation and tubule cells may be affected by kidney disease.[135] Alternatively, iPSCs may be derived from tissue banked cord blood, umbilical cord matrix, or placental amniotic membrane.[134, 135] Compared to ES cells, based on epigenetic patterning, iPSCs are less likely to progress along developmental pathways and are more likely to differentiate back into source tissue.[133, 139] This may be an advantage if the source tissue is healthy kidney. In a study of renal differentiation, fibroblast derived iPSCs lagged behind ES cells when exposed to equivalent inductive factors. It was suggested that continued expression of the embryonic vectors in these iPSCs suppressed differentiation. One possible resolution is to use an alternate method for reprogramming such as synthetic mRNA.[133] Finally, instead of an embryonic state, it may be possible to reprogram cells directly into a kidney precursor population.[131]

Xenogenic Scaffolds

In this research, mouse cells were implanted into rat extracellular matrix scaffolds and with culture showed markers for endothelial progenitors, epithelial cells, and what is likely intermediate mesoderm. It should be considered, however, that this cell response is to a xenogenic scaffold and may be diminished compared to what would have been seen with an allogenic scaffold under equivalent processing and culture conditions. Given this, it may be worthwhile to test cell response with a mouse acellular kidney. In the end though, the only significant way to increase the pool of scaffold tissue for growing replacement kidneys, aside from considerable scaffold fabrication technology improvements, is to use xenograft sources. Therefore, the most viable path to clinical significance is to optimize xenogenic scaffold production and culture conditions for best renal-specific cell response. Furthermore, as optimal scaffold decellularization protocols and culture conditions are likely to shift depending on cell

and tissue sources, early success of this rodent-based research should prompt advancement to human cells on porcine-derived scaffolds, as that pairing is the most likely to be clinically successful.

It has been shown that xenogenic tissue can be decellularized to reduce immunogenicity and allow constructive remodeling upon implantation.[98, 102, 156] This remodeling or "autologization" may consist of tissue-specific host cell infiltration and basement membrane production and is considered a positive interaction of graft tissue and host cells.[147, 221] As an extension of this research, the decellularized rat kidneys implanted with mES cells were examined for mouse ECM production. The analysis showed presence of mouse-specific laminin.[213] This is a positive sign of xenograft remodeling with one caveat. Composition of deposited ECM is reflected by the current phenotype and environment of the cells.[222] If cells of early nephrogenic lineages produce ECM, it may shield signaling cues from the adult matrix, inhibiting further differentiation. In this case, there may be more reliance on culture-based signals. One solution may be to reduce the level of growth factors that promote ECM production, such as TGF-β.[55, 173]

Once cells in the kidney scaffolds have been sufficiently differentiated and arranged in the extracellular scaffold, research can progress to *in vitro* function testing and *in vivo* implantation. One method used by other groups to test organ constructs with intact vasculature is by circulation of blood.[22, 26] In the case of populated kidney scaffolds, the filtrate collected in the ureter could be examined for glucose, amino acids, electrolytes and other molecules to test filtration specificity of the glomerulus and resorptive function of the epithelial cells. The final step in functional analysis is to implant the kidneys by anastomosing to available vasculature, similar to studies of seeded scaffolds that have been generated from heart, liver, and lung.[22-24, 26,

[28] Demonstration of renal function recovery would be a significant advancement for those suffering from renal disease.

CHAPTER 6
MATERIALS AND METHODS

Organ Harvest

All kidneys used in the establishment of a decellularization protocol for renal tissue engineering or any subsequent cell seeding and culture experiments were harvested from male Sprague Dawley rats of at least 2 months of age and 250g weight. The rats were obtained from Harlan Laboratories Inc. (Indianapolis, IN) and housed in the VM-19 Metabolic Building barrier facility at the University of Florida Veterinary Medical Complex under the care of Animal Care Services (Gainesville, FL). All animal use and associated procedures were performed in accordance with the guidelines supplied by the Institute for Lab Animal Research Guide for the Care and Use of Laboratory Animals, regulated by the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee at the University of Florida (UF).

Rat kidneys were harvested according to a modified form of the non-survival procedure described in Aden *et al.* (2005).[143] Animals were anesthetized by chamber induction with 5% isoflurane in 100% oxygen, and then maintained by mask administration of 2-3% isoflurane in 100% oxygen. This method of anesthesia was selected to minimized animal handling and stress prior to the harvest procedure, as well as to better control any discomfort or pain experienced during the procedure. Depth of anesthesia was checked according to righting reflex, reaction to toe pinch as well as overall disposition of the animal (breathing and heart rates). Temperature of the animal was maintained with a circulating water warming pad. After anesthesia was induced, the abdomen was shaved, cleaned, and wiped with isopropyl alcohol. Once the animal was secured in dorsal recumbency, a ventral midline laparotomy was performed and the viscera exteriorized to expose the right kidney. Then, after blunt and sharp dissection, sutures were pre-

placed around the distal cranial mesenteric artery (CMA), and the aorta and vena cava superior to the CMA and inferior to the renal artery. These served to isolate the vascular supply and avoid hemorrhage during cannulation. Before tightening the sutures, 1400 units/kg of heparin sodium was delivered into the peritoneal cavity to reduce coagulation in the organ. Once the vessels were ligated, an ophthalmic 19G Park Irrigating Cannula (Bausch & Lomb Surgical, Inc., Rancho Cucamonga, CA) was quickly introduced into the cranial mesenteric artery and directed across the aorta and into the right renal artery. Sutures were then used to secure the cannula to the renal artery. Once anchored, the renal vein was nicked and the kidney was flushed with 40ml of a 0.9% saline solution containing sodium nitroprusside, a vasodilator; during the flush, the kidney was observed for blanching to assure a complete rinse of blood. After the rinse, the attaching vessels and ureter were cut and the kidney was removed en bloc from the retroperitoneal space. Then, while under anesthesia, the rat was euthanized with an intracardiac injection of 120 mg/kg sodium pentobarbital with confirmation by thoracotomy.

On occasion, variant anatomy or damage of the CMA necessitated an alternate route of cannulation, and it has been achieved with aortic cannulation both superior and inferior and directed into to the renal artery. Another procedural variation was the addition of a ureteral cannula, PE 10 tubing, which was used for filtrate collection or retrograde cell delivery.

Organ Decellularization

Organ Perfusion

Two perfusion systems were used to decellularize kidneys. In both cases, the harvested kidney, suspended by the renal artery and attached cannula, were affixed to the system by a luer fitting. Prescribed decellularization solutions were then perfused into the kidney through the renal artery and drained from the renal vein or ureter into a collection receptacle for disposal or

recirculation. Perfusates were delivered at room temperature with a pressure maintained between 90 and 110 mmHg, within rat physiologic limits.

The first perfusion system used was the LifeSustainer 1000 automated organ perfusion workstation (LifeSystems, Redmond, WA).[144] It was a system designed to maintain viability of an organ ex vivo for scientific research and monitors pH, fluid pressure, as well as oxygen and carbon dioxide saturation. In addition, it has controls for regulation of temperature and perfusion with flow rate or pressure regulation. Once the kidneys were fixed to the machine, perfusate solutions were pulled from prepared bottles and circulated according to the prescribed decellularization protocol.

The second perfusion system was designed and constructed in house and drastically improved pressure regulation and air entrapment in fluid lines. In addition, it included a large solution reservoir, accessible plumbing, a .8/.2μm Polycap ™ TC high flow inline filter for debris entrapment (Whatman, Inc., Piscataway, NJ), and capacity for decellularization of two kidneys simultaneously. A diagram and apparatus images are included in Appendix A (Figures A-1 and A-2).

Decellularization Protocols

Harvested kidneys, once attached to a perfusion system, were subject to a series of solutions delivered into the vasculature with the express goal of removing all cellular material in a progressive manner without causing blockage within the intricate vascular and tubular networks or disruption of the extracellular matrix. The following protocols were based on earlier works from Brendel, Meezan, and Nagle (1979, 1980), who desired to isolate the basement membrane of the kidney and other organs for study of morphology, permeability, and chemical composition.[141, 142] It was the purpose of this work to evaluate and modify the original decellularization protocols so as to produce an acellular whole organ kidney amenable to cell

seeding and other tissue engineering or regenerative applications. The protocols included use of the nonionic detergent Triton X-100 (Sigma-Aldrich, St. Louis, MO) for solubilization of hydrophobic cell membranes, cell lysis, and washing of resulting debris. Additives were used such as sodium chloride to osmotically disrupt nuclear membranes or calcium chloride (Fisher Scientific, Hampton, NH) and magnesium sulfate (Fisher) to improve membrane permeability and activate endogenous nucleases for digestion of bulky nuclear material. DNA was enzymatically degraded with a buffered solution of 2000 Kunitz units/mg deoxyribonuclease 1 (DNase) from bovine pancreas (Sigma). The buffer constituents sodium acetate, magnesium chloride, and sodium chloride were all acquired from Fisher Scientific. Ionic detergents such as sodium deoxycholate (NaDC) and sodium dodecyl sulfate (SDS) were optionally used to remove any debris or non-soluble cellular material. Finally, deionized water was used as an intermediate or final rinsing step and may have exerted osmotic stress on cell membranes. Each of the detergent solutions contained .05% sodium azide as an antimicrobial and all solutions were aqueous. After a final rinse, if a kidney was intended for cell injection and culture, 50cc of Dulbecco's modified Eagle's medium (DMEM) was perfused and the cannula was sprayed with 70% ethanol before removal from the decellularization apparatus.

The following tables describe the decellularization protocols used for preparing an acellular kidney tissue engineering scaffold. Appended to each is a description of modifications.

Decellularization protocol: VBM. This protocol (Table 6-1) was based on Brendel and Meezan's VBM preparation (Brendel, 1980).[142]

Decellularization protocol: VBM Mod 1. This protocol was identical to *VBM* (Table 6-1) except with reduction of the Triton X-100 detergent concentration in step 1 to 0.5%. This

Table 6-1. VBM decellularization protocol

Step	Dur	Rec	Solution
1	24h	N	2% v/v Triton X-100
2	24h	Y	2% v/v Triton X-100
3	2h	N	De-ionized water
4	15m	N	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
5	4h	Y	2% v/v Triton X-100
6	1h	N	De-ionized water
7	2h	N	20% v/v ethanol
8	1h	N	De-ionized water

Total duration: 58h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

Table 6-2. Kidney Mod 1 decellularization protocol

Step	Dur	Rec	Solution
1	3h	N	0.5% v/v Triton X-100
2	3h	Y	0.5% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$
3	2h	N	De-ionized water
4	4h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
5	3h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$
6	3h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
7	2h	N	De-ionized water
8	4h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
9	2h	N	De-ionized water
10	2h	Y	4% w/v sodium deoxycholate
11	4h	N	De-ionized water

Total duration: 58h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

change, motivated by Brendel and Meezan's kidney-specific protocol (1979), was thought to minimize sudden release of proteinaceous debris and subsequent clogging of tubules.[141]

Decellularization protocol: Kidney Mod 1. This was the first protocol used with the elevated reservoir perfusion apparatus (Appendix A-1). Steps were incorporated from the acellular perfused kidney protocol (Brendel, 1979) to improve debris removal and rupture of intact nuclear envelopes (Table 6-2). This included changing perfusion durations, the addition of calcium chloride, magnesium sulfate, and sodium chloride to the detergent solutions, and use of

Table 6-3. Kidney Mod 2 decellularization protocol

Step	Dur	Rec	Solution
1	3h	N	0.5% v/v Triton X-100
2	9h	Y	0.5% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$
3	2h	N	De-ionized water
4	4h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
5	6h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$
6	16h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
7	2h	N	De-ionized water
8	4h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
9	2h	N	De-ionized water
10	8h	Y	4% w/v sodium deoxycholate
11	6h	N	De-ionized water

Total duration: 62h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

the ionic detergent sodium deoxycholate as an extra cleaning step. Also, a second DNase step was added to reduce any buildup of viscous DNA. Finally, there was removal of ethanol to avoid any fixation.

Decellularization protocol: Kidney Mod 2. The duration of detergent perfusion steps, both nonionic and ionic, were increased from *Kidney Mod 1* to test for better cell debris removal (Table 6-3). Specifically, step 6, the most aggressive nonionic detergent step, was significantly increased to a duration commensurate with the acellular perfused kidney protocol (Brendel, 1979).[141]

Decellularization protocol: Graduated 3%. This protocol was developed to offer a slow, graduated increase in detergent concentration with late addition of additives (calcium chloride, magnesium sulfate, and sodium chloride). There was also only one DNase step (Table 6-4).

Decellularization protocol: Graduated 10%. This protocol was similar to *Graduated 3%*, except for more aggressive detergent concentrations (Table 6-5).

Table 6-4. Graduated 3% decellularization protocol

Step	Dur	Rec	Solution
1	3h	N	0.1% v/v Triton X-100
2	6h	Y	0.1% v/v Triton X-100
3	9h	Y	0.5% v/v Triton X-100
4	9h	Y	3% v/v Triton X-100
5	9h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$
6	9h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
7	2h	N	De-ionized water
8	6h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
9	3h	N	De-ionized water
10	9h	Y	4% w/v sodium deoxycholate
11	4h	N	De-ionized water

Total duration: 69h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

Table 6-5. Graduated 10% decellularization protocol

Step	Dur	Rec	Solution
1	3h	N	0.5% v/v Triton X-100
2	7h	Y	0.5% v/v Triton X-100
3	10h	Y	3% v/v Triton X-100
4	10h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
5	10h	Y	6% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
6	10h	Y	10% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
7	2h	N	De-ionized water
8	6h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
9	2h	N	De-ionized water
10	10h	Y	4% w/v sodium deoxycholate
11	4h	N	De-ionized water

Total duration: 74h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

Decellularization protocol: Kidney Extended. This protocol resembled *Kidney Mod 2*, yet with a very long Triton X-100 with additives detergent step interrupted by a DNase step (Table 6-6).

Decellularization protocol: Kidney Extended 2. This protocol was identical to *Kidney Extended* except for the removal of the first DNase step and rinse, and a lengthening of the Triton X-100 with additives step to gain an equivalent total duration (Table 6-7).

Table 6-6. Kidney Extended decellularization protocol

Step	Dur	Rec	Solution
1	3h	N	0.5% v/v Triton X-100
2	24h	Y	3% v/v Triton X-100
3	40h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
4	6h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
5	2h	N	De-ionized water
6	64h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
7	6h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
8	2h	N	De-ionized water
9	20h	Y	4% w/v sodium deoxycholate
10	4h	N	De-ionized water

Total duration: 171h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

Table 6-7. Kidney Extended 2 decellularization protocol

Step	Dur	Rec	Solution
1	3h	N	0.5% v/v Triton X-100
2	24h	Y	3% v/v Triton X-100
3	112h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
4	6h	Y	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
5	2h	N	De-ionized water
6	20h	Y	4% w/v sodium deoxycholate
7	4h	N	De-ionized water

Total duration: 171h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

Decellularization protocol: Kidney NaDC. This protocol was another combination of the Brendel *et al.* decellularization protocols (1979, 1980) that incorporated the detergent additives and ionic detergent from the former and the convenient 24 hour-based timing of the second protocol (Table 6-8).[141, 142] Also, in contrast to the previous protocols, the initial non-recirculating step was changed to recirculating due to reliance on the inline filter to capture cell debris.

Table 6-8. Kidney NaDC decellularization protocol

Step	Dur	Rec	Solution
1	21h	Y	3% v/v Triton X-100
2	27h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
3	2h	N	De-ionized water
4	22h	N	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
5	1h	N	De-ionized water
6	23h	N	3% v/v Triton X-100
7	21h	Y	4% w/v sodium deoxycholate
8	3.5h	N	De-ionized water

Total duration: 120.5h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

Table 6-9. Kidney SDS decellularization protocol

Step	Dur	Rec	Solution
1	21h	Y	3% v/v Triton X-100
2	27h	Y	3% v/v Triton X-100, 5mM $CaCl_2$, 5mM $MgSO_4$, 1M NaCl
3	2h	N	De-ionized water
4	22h	N	50mM sodium acetate, 10mM $MgCl_2$, 100mM NaCl, .0025% DNase (pH 5.0)
5	1h	N	De-ionized water
6	23h	N	3% v/v Triton X-100
7	21h	Y	4% w/v sodium dodecyl sulfate
8	3.5h	N	De-ionized water

Total duration: 120.5h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

Decellularization protocol: Kidney SDS. This protocol was identical to the *Kidney NaDC* protocol except for replacement of ionic detergent with an equivalent concentration of SDS (Table 6-9).

Decellularization protocol: 1-Day SDS. This protocol tested if the ionic detergent sodium dodecyl sulfate was the primary determinant of decellularization success by removing all steps in the *Kidney SDS* protocol that precede SDS treatment. (Table 6-10).

Table 6-10. Kidney 1-Day SDS decellularization protocol

Step	Dur	Rec	Solution
7	24h	Y	4% w/v sodium dodecyl sulfate
8	3.5h	N	De-ionized water

Total duration: 27.5h. Key: dur, length of perfusion; rec, perfusate is recirculated and filtered; solution, perfusate recipe.

In Vitro Embryonic Stem Cell Engraftment and Culture

Embryonic Stem Cells

Green fluorescent murine embryonic stem (mES) cells (B5/EGFP, courtesy of Andras Nagy) were used to test the ability of the acellular kidney scaffolds to direct differentiation of implanted pluripotent stem cells.[194] These cells have been well characterized and the persistence of fluorescence through cell division and differentiation allowed visualization not only of initial cell seed distribution but the subsequent cell migration and grouping patterns over the length of the culture period.

The murine B5/EGFP ES cells were cultured according to Singh *et al.* (2007).[223] The mES cells were maintained in an undifferentiated state on gelatin-coated dishes in Knock-out DMEM (Life Technologies BRL, Grand Island, NY) containing 10% knockout serum replacement (Life Technologies BRL), 1% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 25mM HEPES (Life Technologies BRL), 300 μM monothioglycerol (Sigma), and 1000 units/ml recombinant mouse LIF (ESGRO Chemicon, Temecula, CA). To prepare the cells for injection, the undifferentiated ES cells were dissociated using 0.25% trypsin/EDTA (Life Technologies BRL). Then, they were suspended in a solution devoid of LIF, which was also used as the growth media that would permit differentiation in all three of the scaffold incubation protocols described below: Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Life

Technologies BRL), 2.5μg/ml Fungizone® antimycotic (Life Technologies BRL), and 300 μM monothioglycerol. All cultures in this study were maintained at 37 °C in 5% CO_2. The B5/EGFP ES cell line was maintained by Dr. Takashi Hamazaki in the laboratory of Dr. Naohiro Terada.

Cell Engraftment and Culture

Immediately following decellularization, kidneys that were allocated for mES cell seeding were perfused with 20ml of DMEM (Fisher Scientific) and placed in a sterile container for transport. Then under sterile conditions, $2x10^6$ ES cells suspended in growth medium were slowly injected into the arterial or ureteral cannula so as not to cause an ECM rupture from pressure buildup. One of three scaffold culture methods was then followed, whole kidney static culture, whole kidney perfused culture, or sectioned scaffold culture as described below. Scaffolds were cultured for durations of 6, 10, and 14 days, with additional time points taken at 3, 4, or 8 days as tissue was available. This range of time points was selected to capture any transient differentiation patterns that may parallel normal murine nephrogenic development. Frequency of media change is described within each culture method; however, at first change in all cases, the Fungizone® antimycotic was reduced to a maintenance level of 0.25μg/ml. During scaffold culture, gross cell localization, migration, and grouping tendencies were monitored through GFP expression by fluorescence microscopy (IX70; Olympus, Tokyo, Japan).

Whole scaffold static culture. The initial culture method used was simple submersion of the seeded scaffold into a 50ml conical culture tube. Growth media was refreshed every 2-3 days.

Perfused organ culture. In an effort to improve viability and provide a more suitable microenvironment, seeded acellular scaffolds were placed in a perfused culture system. This sealed system was specifically designed to allow independent adjustment of mean pressure and pulse rate, with a pulsatile pressure differential of between 20 and 30 mmHg. This allowed

gradual increase of mean pressure over time with maintenance of periodicity. Typically, the scaffolds were injected with cells and affixed to the apparatus with submersion in media, but circulation of the culture was not initiated for 12 hours. This method gave the cells time to adhere to the ECM before being exposed to shear stress. Medical grade sterile gases (5% CO_2 air mix, Praxair, Inc., Danbury, CT) were delivered to the culture chamber and media reservoir, to maintain media at pH 7.4. Given the high volume of the circulated media reservoir, growth media was only changed every 6-7 days. An apparatus diagram and images are shown in the appendices (Figure A-3 and A-4).

Sectioned scaffold culture. The final seeded scaffold culture employed involves sectioning the kidney in thick sections so that multiple time points can be accomplished with a single decellularized organ preparation. In addition, it was assumed that sectioning of the kidney would lessen any diffusional barriers that developed after continued cell growth. Initially, a fresh scaffold was injected with cells and left to sit in a deep cell culture dish (60cm) for 24 hours to let the cells adhere. Then the scaffold was sectioned transversely in 2-3 mm increments, with samples placed in a 12-well cell culture dish. Sections which retained the primary functional areas of the kidney, cortex, outer medulla, and inner medulla were used for data time points.

Embryoid Bodies

Embryoid bodies were cultured at analogous time points to the seeded kidney scaffold cultures. This allowed ES cells to multiply and differentiate without influence of contacting extracellular matrix. This experimental control was useful in immunohistochemical and gene expression comparisons. Using the differentiation medium described above, ES cells were cultured for 2 days by the hanging-drop method (2×10^3 ES cells per each 30 µl drop). Afterward, embryoid bodies in hanging drops were transferred to suspension culture in 100-mm petri dishes and incubated for 3,6,10 and 14 days. At the designated time point, EBs were

encapsulated in HistoGel™ before tissue processing (as described in histochemistry). The culture of embryoid bodies was performed by Dr. Takashi Hamazaki in the laboratory of Dr. Naohiro Terada.

Histochemistry

Tissue Preparation

Most of the freshly prepared decellularized kidneys and cell-seeded and cultured scaffolds were prepared for histological evaluation. First, the kidneys were submerged in 10% neutral buffered formalin for 24 hours. The NBF was Richard-Allan Scientific brand and distributed by Fisher Scientific. Afterward, the kidney scaffolds were transferred to phosphate buffered saline and submitted to the Molecular Pathology & Immunology Core at the University of Florida for paraffin embedding. Once embedded, 5μm transverse sections were taken from the midline, warmed to 42 ºC in a water bath, placed on Superfrost Plus positively charged slides (Fisher Scientific), and dried overnight at room temperature using a ductless enclosure. Slides were then earmarked for H&E staining or IHC; those protocols are described below. Completed histology slides were observed with an Olympus BX51 Microscope and photographed using an Optronic Digital Camera with Magnafire 3.1 software.

Hematoxylin and Eosin Staining

The primary method for tissue evaluation was microscopic inspection of hematoxylin and eosin (H&E) stained paraffin-embedded tissue. The slide-mounted tissue sections were deparaffinized, rehydrated, stained, and dehydrated according to Table 6-10. Steps include use of Hematoxylin 7211 for blue staining of cell nuclei, Clarifier 1 to remove residual hematoxylin bound to the slide, Bluing Reagent to enhance the blue color, and Eosin-Y to stain proteins pink.

These reagents were all Richard-Allan Scientific brand. Once the tissue was stained, coverslips were applied to the slides using Cytoseal XYL (Richard-Allan Scientific). All

Table 6-11. Hematoxylin and Eosin Staining Protocol

Step	Duration	Solution
1	5m	Xylene
2	5m	Xylene
3	2m	100% ethanol
4	2m	100% ethanol
5	1m	95% ethanol
6	1m	95% ethanol
7	1m	Distilled water
8	2m 15s	Hematoxylin
9	1m	Distilled water
10	1m	Distilled water
11	1m	Clarifier
12	1m	Distilled water
13	1m	Bluing Reagent
14	1m	Distilled water
15	1m	80% ethanol
16	1m 30s	Eosin-Y
17	1m	95% ethanol
18	1m	95% ethanol
19	1m	100% ethanol
20	1m	100% ethanol
21	1m	xylene
22	1m	xylene
23	1m	xylene

Slides were spaced on a rack and sequentially submerged in a series of dishes containing the solution as indicated above.

hematoxylin and eosin histological staining was performed at the UF Stem Cell Program, Core Histology Laboratory.

For the purpose of scaffold evaluation, each of the samples was scrutinized for preservation of extracellular matrix morphology in the cortex, outer medulla, and inner medulla of the kidney scaffold. In addition, it was noted if there were intact cells, nuclei, stained DNA aggregates, or other un-cleared cellular debris. For analysis of seeded and cultured kidney scaffolds, the slides were inspected for cell distribution, grouping, morphology, ECM association, and appearance of apoptosis.

Table 6-12. Immunohistochemical Antibodies

Target Protein	Species	Company	Cat #	Dilution	Retrieval
Cleaved Caspase-3	Rabbit	Cell Signaling Tech	9661	1:50	Dako
Collagen IV	Rabbit	Abcam	AB13966	1:500	Prot K
GFP	Rabbit	Abcam	290	1:500	Citrate
Ki67	Rabbit	Leica	NCL-KI67P	1:2000	Dako
Ksp-cadherin	Mouse	Invitrogen	35-4700	1:200	None
Laminin	Rabbit	Abcam	AB11575	1:25	Prot K
Oct4	Rabbit	Abcam	AB20650	1:1000	Hi pH
Pan-cytokeratin*	Mouse	Ventana	760-2135		
Pax-2	Rabbit	Invitrogen	71-6000	1:200	Citrate
WT1*					

*IHC performed by Shands Medical Laboratories at Rocky Point (Gainesville, FL).

Retrieval methods: citrate, sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 25 mins at RT; Dako, Dako Target Retrieval Solution (Dako North America, Inc., Carpinteria, CA) for 20 mins at 95ºC and 20 mins at RT; Hi pH, distilled water (pH 10.0) for 25 mins at 95ºC and 10 mins at RT; Prot K, proteinase K enzymatic solution.

Company details: Abcam, Inc. (Cambridge, MA); BD Pharmingen (San Diego, CA); Cell Signaling Technology, Inc. (Danvers, MA); Invitrogen Corporation (Carlsbad, CA); Leica Microsystems Inc. (Buffalo Grove, IL); Ventana Medical Systems, Inc. (Tucson, Arizona).

Immunohistochemistry

Immunohistochemistry was used to detect retention of extracellular proteins in rat decellularized kidney scaffolds and to identify patterns of cell division, apoptosis, and differentiation of implanted murine embryonic stem cells. In addition, embryoid bodies and embryonic kidneys (E17.5) were used to provide experimental controls. As cell implantation was on a xenogenic scaffold, both rat and mouse tissue was used to test for antibody cross-reactivity or non-specific binding. All IHC, with exception of the antibodies indicated in Table 6-11, was performed at the UF Stem Cell Program, Core Histology Laboratory (Gainesville, FL). The stains were implemented using the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) according to manufacturer's instructions. It employed an enzyme-based (immunoperoxidase) reaction and the chromogen 3,3'-Diaminobenzidine (DAB) to develop color. To initiate the process, tissue slides were deparaffinized and hydrated according to Table

Table 6-13. Slide Pretreatment for Immunohistochemistry

Step	Duration	Solution
1	5m	Xylene
2	5m	Xylene
3	2m	100% ethanol
4	2m	100% ethanol
5	10m	3% hydrogen peroxide in methanol
6	3m	95% ethanol
7	1m	70% ethanol
8	1m	Distilled water
9	1m	Distilled water

Slides were spaced on a rack and sequentially submerged in a series of dishes containing the solution as indicated above.

6-12. Included was a step to quench endogenous peroxidase activity (3% hydrogen peroxide). Next, an antigen unmasking or retrieval step was performed, the specific method indicated and described on Table 6-11. The steps proceeded according to instruction, with TBS-T (Tris-buffered saline + 0.1% Tween) buffer and the use of an avidin/biotin blocking step (Avidin/Biotin Blocking Kit, Vector Laboratories). Primary antibodies (Table 6-11) with listed dilutions were incubated overnight at 4ºC, except for GFP, which was incubated for 1 hour at 4ºC. Secondary antibodies were incubated for 30 minutes. Finally, after the slides were developed and counterstained with hematoxylin (Hematoxylin 7211, Richard-Allan Scientific), the slides were covered using Cytoseal XYL (Richard-Allan Scientific) mountant.

Scanning Electron Microscopy

Scaffold morphology, including quality of decellularization and basement membrane continuity, was evaluated using the scanning electron microscopy facilities at the Major Analytical Instrumentation Center, University of Florida. Sample preparation and equipment operation was performed by Dr. Brad Willenberg. The freshly decellularized kidneys were fixed overnight in 2.5% glutaraldehyde at room temperature, freeze-dried, then fractured on the transverse, midline plane. The samples were mounted on an aluminum stub (SPI Supplies, West Chester, PA) and sputter coated with carbon (Ion Equipment Corp., Santa Clara, CA). The

prepared samples was analyzed using a JEOL JSM-6400 SEM (JEOL USA, Peabody, MA) equipped with an Oxford energy dispersive spectroscope (EDS) system and a LINK ISIS software package version 3.35 (Oxford Instruments USA, Concord, MA).

APPENDIX A
DECELLULARIZATION AND PERFUSION CULTURE APPARATUSES

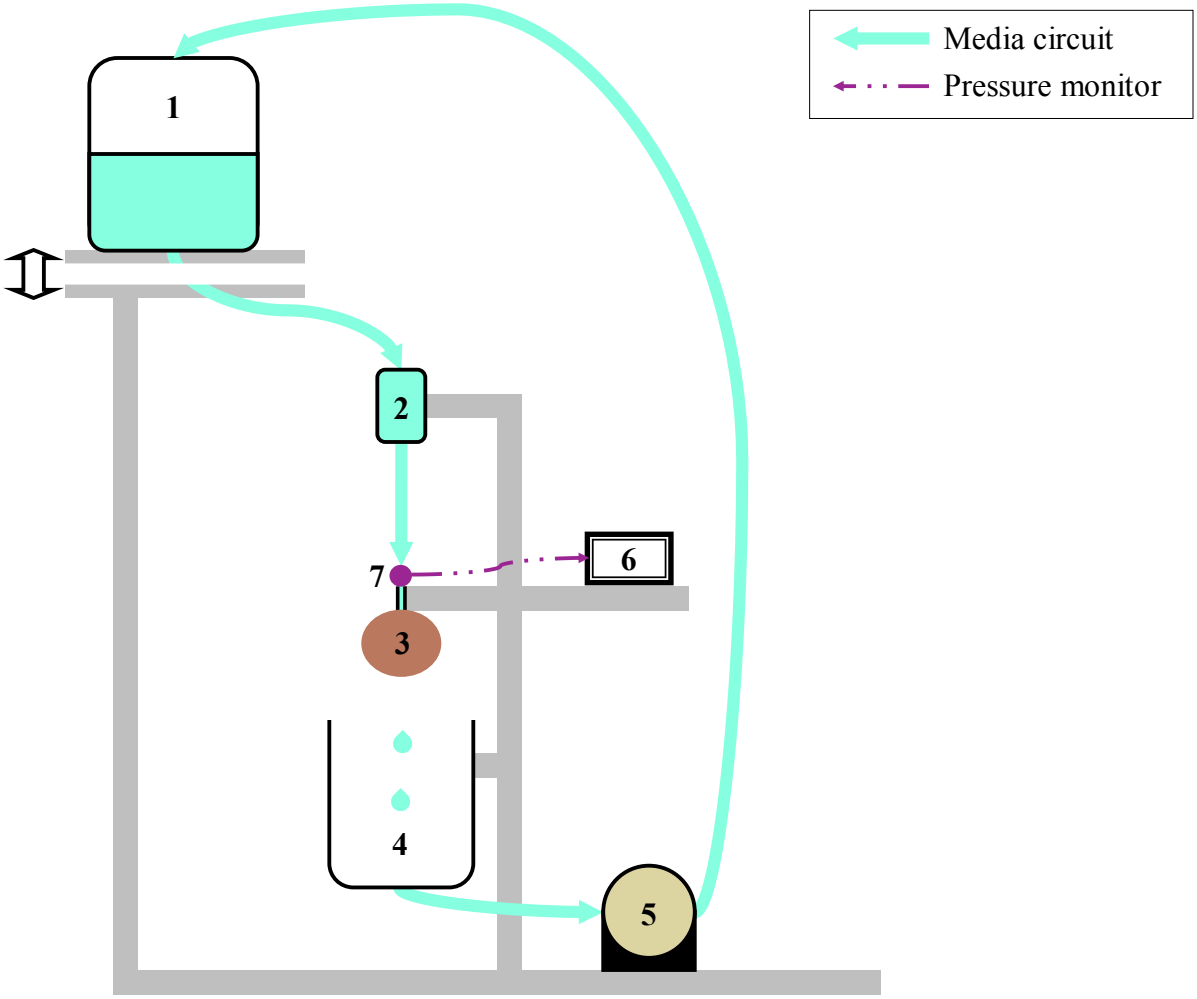

Figure A-1. Decellularization apparatus diagram. This system was designed to perfuse an organ at constant pressure. The pressure was determined by the height of the solution in an elevated reservoir relative to the organ. The pressure was adjusted by raising or lowering the reservoir bottle. The decellularization solution in the reservoir bottle (1) passed through a .2/.8 micron inline fluid filter (2) and was delivered into the cannulated renal artery of the kidney (3). The solution perfused the kidney, emptied out of the ureter or renal vein, and was collected in a beaker (4). A peristaltic pump (5) then drew out the perfused fluid and either directed it to a waste container or recirculated it back to the elevated reservoir bottle. Pressure was monitored (6) at the arterial cannula (7). Leaks in the descending fluid line or an insecure arterial cannula caused increased flow and corresponding pressure drop. Flow through the kidney was also affected by type of solution and decellularization progress. Progressive removal of cellular material during decellularization lowered fluid resistance. Despite these factors, and the variable reservoir fluid heights, pressure was typically maintained to within +/- 10% of 100 mmHg.

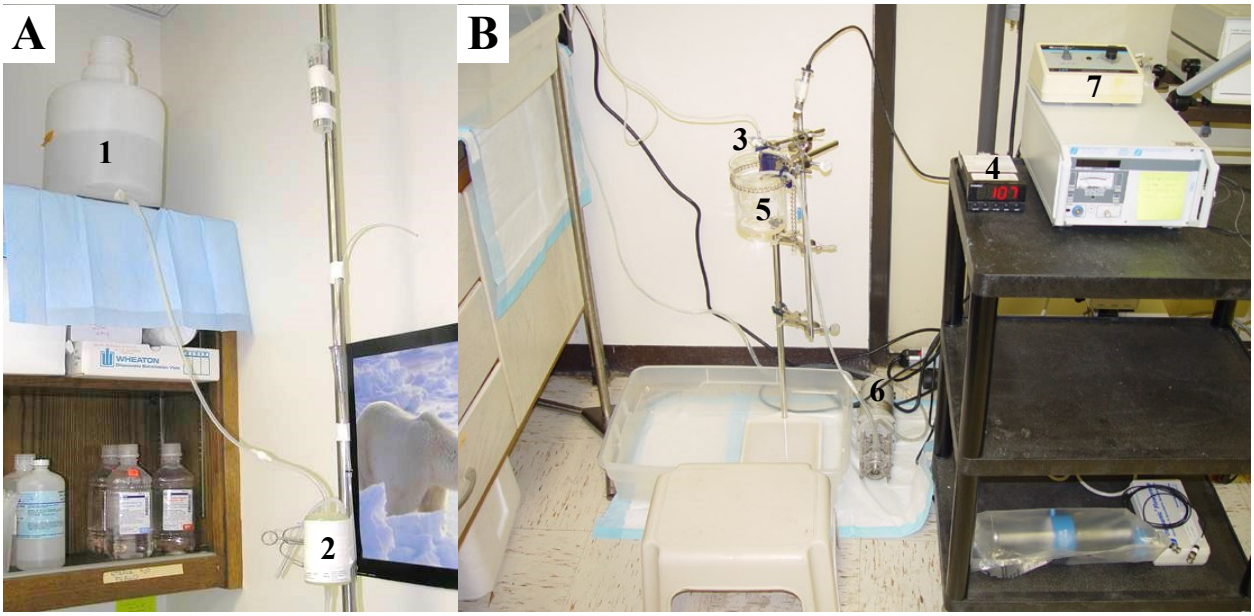

Figure A-2. Pressure based organ perfusion apparatus for decellularization. A) Perfusate reservoir (1) with descending fluid line that passes through a .2/.8µ filter (2). B) Continuation of descending fluid line terminating into a Luer fitting (3) that allows both organ attachment and pressure measurement (4). A beaker (5) collects perfusate that has passed through the organs and optionally returns it to the elevated reservoir with a peristaltic pump (6). The pump controller is also pictured (7). As pictured, there is no kidney being decellularized and the reservoir bottle return line is not attached. Tygon 3350 tubing was used for minimal extractables and ultra-smooth inner bore for reduced potential for particle entrapment.

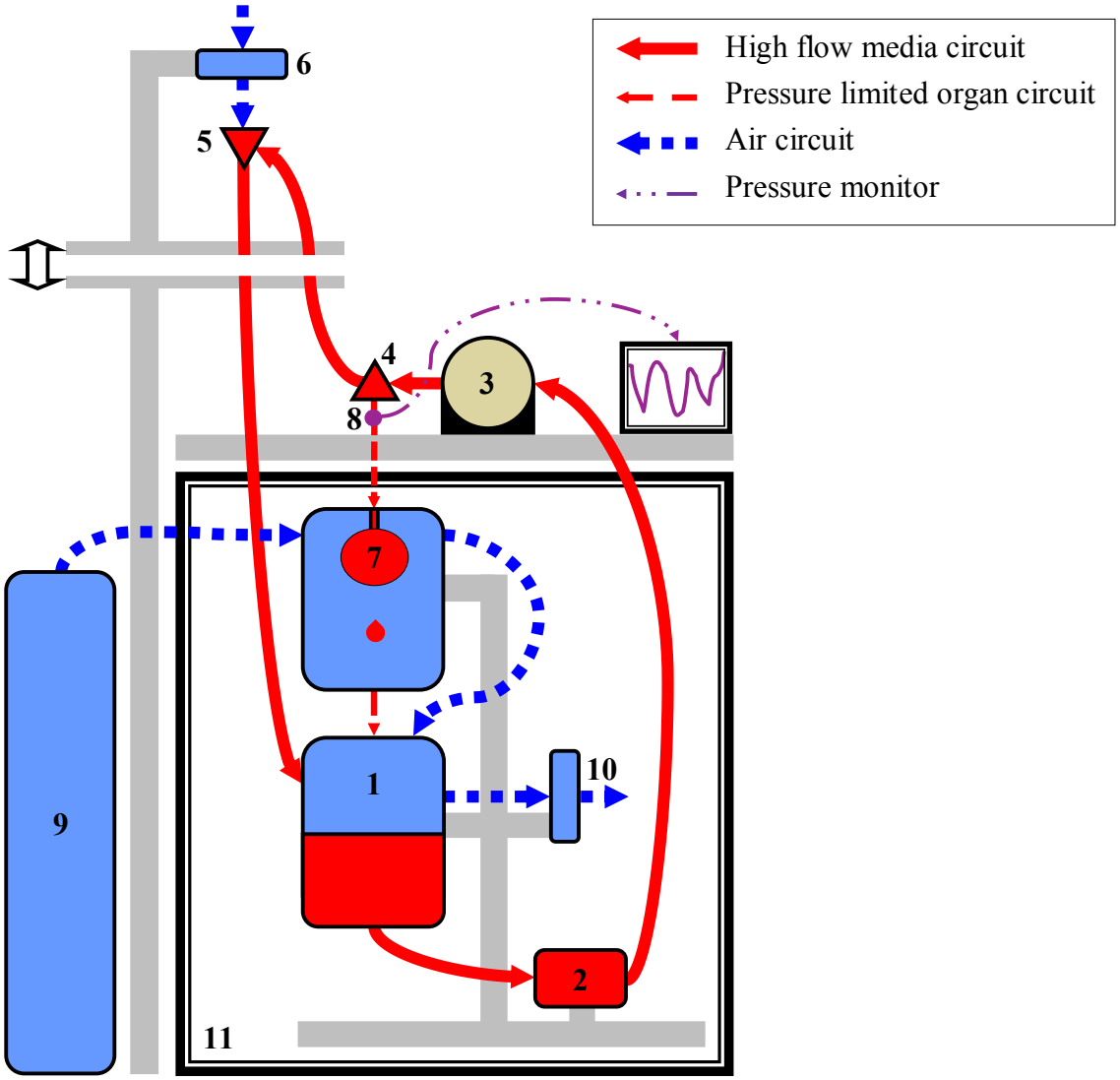

Figure A-3. Perfusion culture apparatus diagram. Primary high flow media circuit: media was drawn from a reservoir (1), passed through a .2/.8 micron filter (2), pulled through a variable-speed peristaltic pump (3), and driven through both a tubing splitter (4) and simple elevated assembly (5 and 6) before returning to the reservoir (1). The elevated assembly, a tubing connector (5) vented to the air through a filter (6), allowed media pushed to a prescribed height to mix with the air and passively fall to the reservoir. The tubing splitter adjacent to the pump (4) let media travel through the kidney (7) at pressure determined by the height of the vertical media column. Media was not solely forced into the kidney so there was little risk of ECM rupture; the flow rate through the kidney was therefore dependent on resistance in the kidney. The pressure waveform was monitored (8) in close proximity to the kidney (diagram is not precise in arrangement or scale). Finally, media that was passed through the kidney collected in the media reservoir (1). To maintain sterility and improve gas diffusion in media, tank-delivered (9) sterile biomedical grade gas (5% CO_2 in air mix) was gently passed through the organ chamber (7) and media reservoir (1). An air filter (10) was used on exhaust to assure sterility. To control temperature, the organ chamber and media reservoir were placed within an incubator (11).

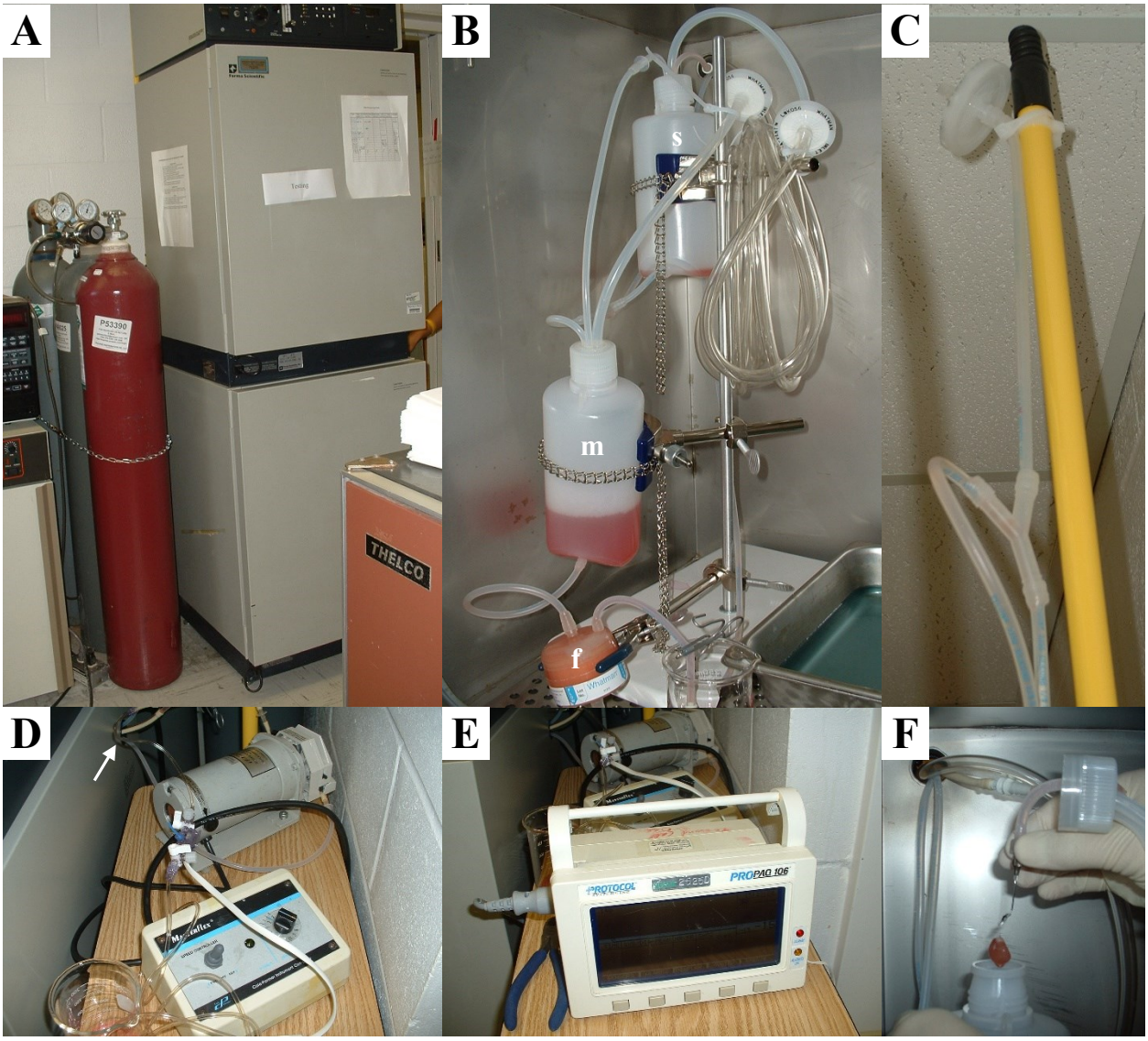

Figure A-4. Organ perfusion bioreactor. This system was designed to circulate media through a seeded acellular organ. Tubing, connectors, and bottles were all reusable and sterilizable. Most of the plumbing could be assembled in a biological hood, and only a couple of connections outside the hood were necessary. A) Incubator with adjacent biomedical gas tank. B) Interior of incubator containing an upper scaffold perfusion bottle (s), a lower media reservoir (m), and a media filter (f). The media filter prevented cells from traveling though circulation. Also visible are the white disc air filters. C) Elevated assembly that controlled pressure to kidney. D) Pump and controller located behind incubator. Fluid lines were passed through the ventilation port on the back of the incubator (arrow). E) A tubing split connector (hidden) granted fluid access to a pressure transducer in proximity to the kidney. The disposable TruWave pressure transducers were gifted from Edwards Lifesciences (Irvine, CA). Pressure waveforms were observed using a donated monitor from Clinical Engineering at Shands Hospital (Gainesville, FL). F) Placement of seeded acellular kidney into the perfusion chamber.

APPENDIX B
SUPPLEMENTAL HISTOLOGY

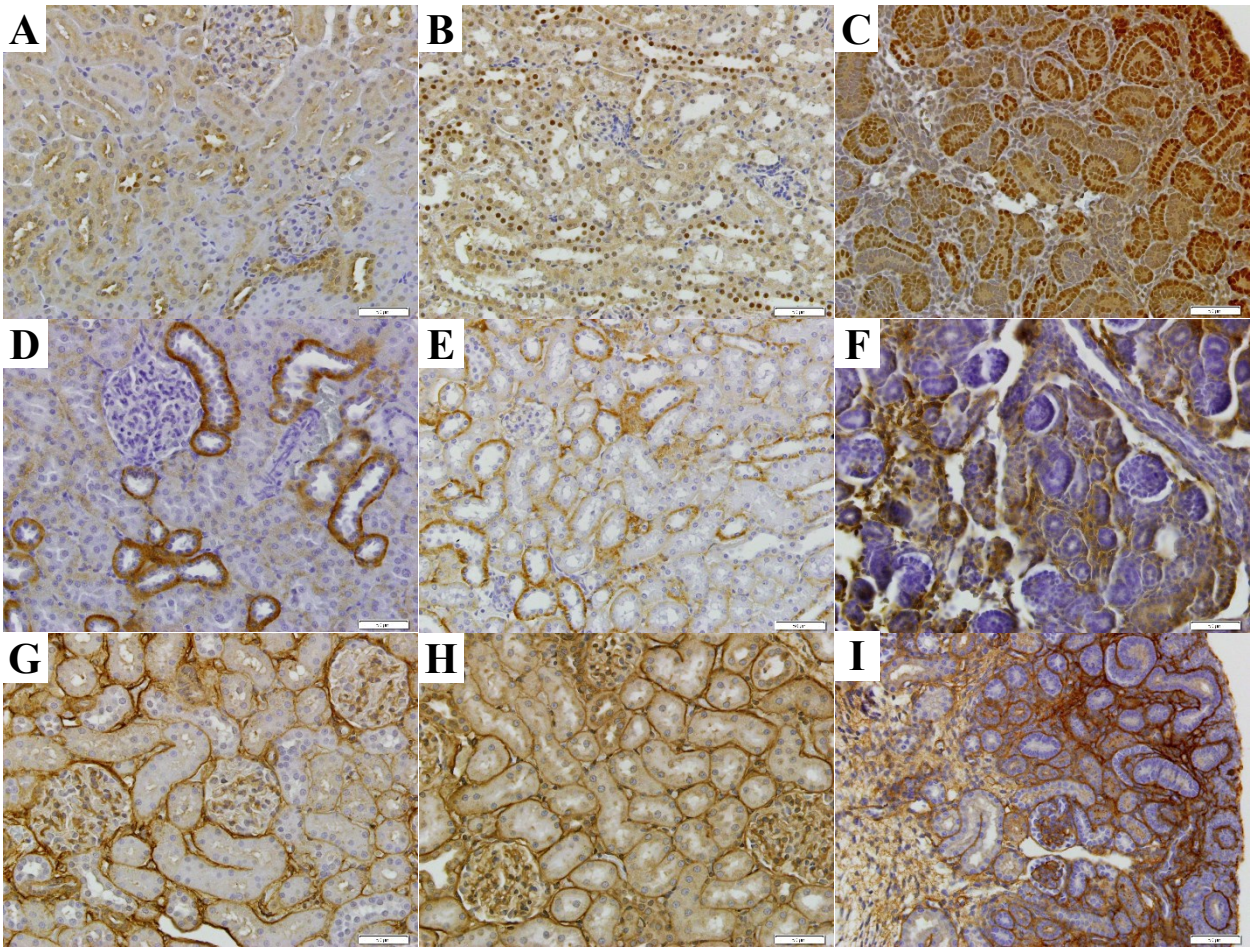

Figure B-1. Reference immunohistochemical comparison of normal rodent kidney tissue (40x magnification, bars denote 50µm). Pax-2 in A) adult rat, B) adult mouse, and C) embryonic mouse at 17.5 days post conception; Ksp-cadherin in D) adult rat, E) adult mouse, and F) embryonic mouse at 17.5 days post conception; G) Collagen IV in adult rat; H) laminin in adult rat; and I) pan Cytokeratin in embryonic mouse at 17.5 days post conception.

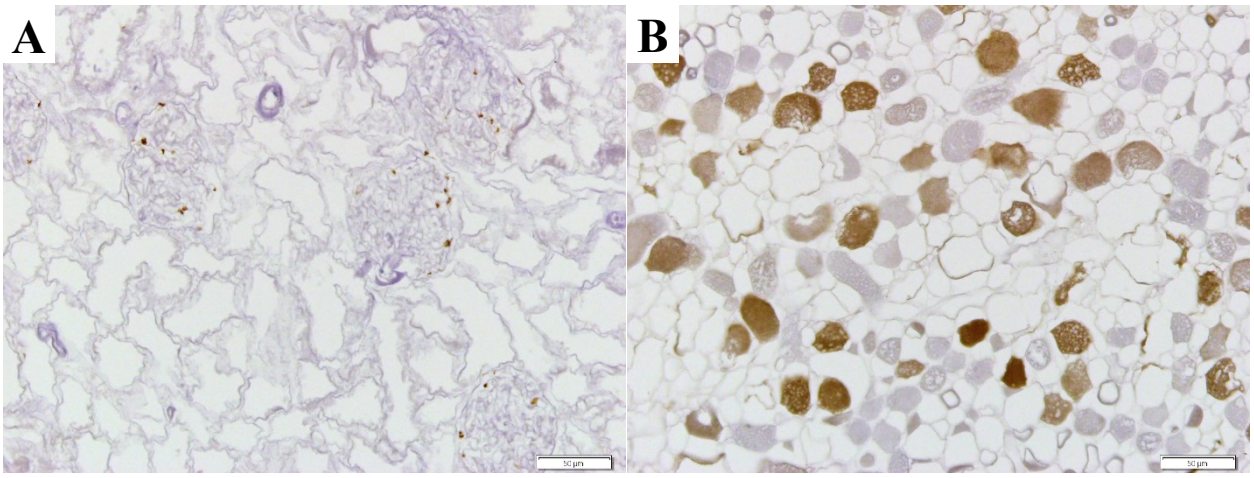

Figure B-2. Localization patterns of unremoved cellular proteins post *Kidney NaDC* decellularization using IHC (40x magnification, bars denote 50µm). A) WT1 in expected glomerular location, and B) possible diffuse Ksp-cadherin protein in unremoved proteinaceous debris.

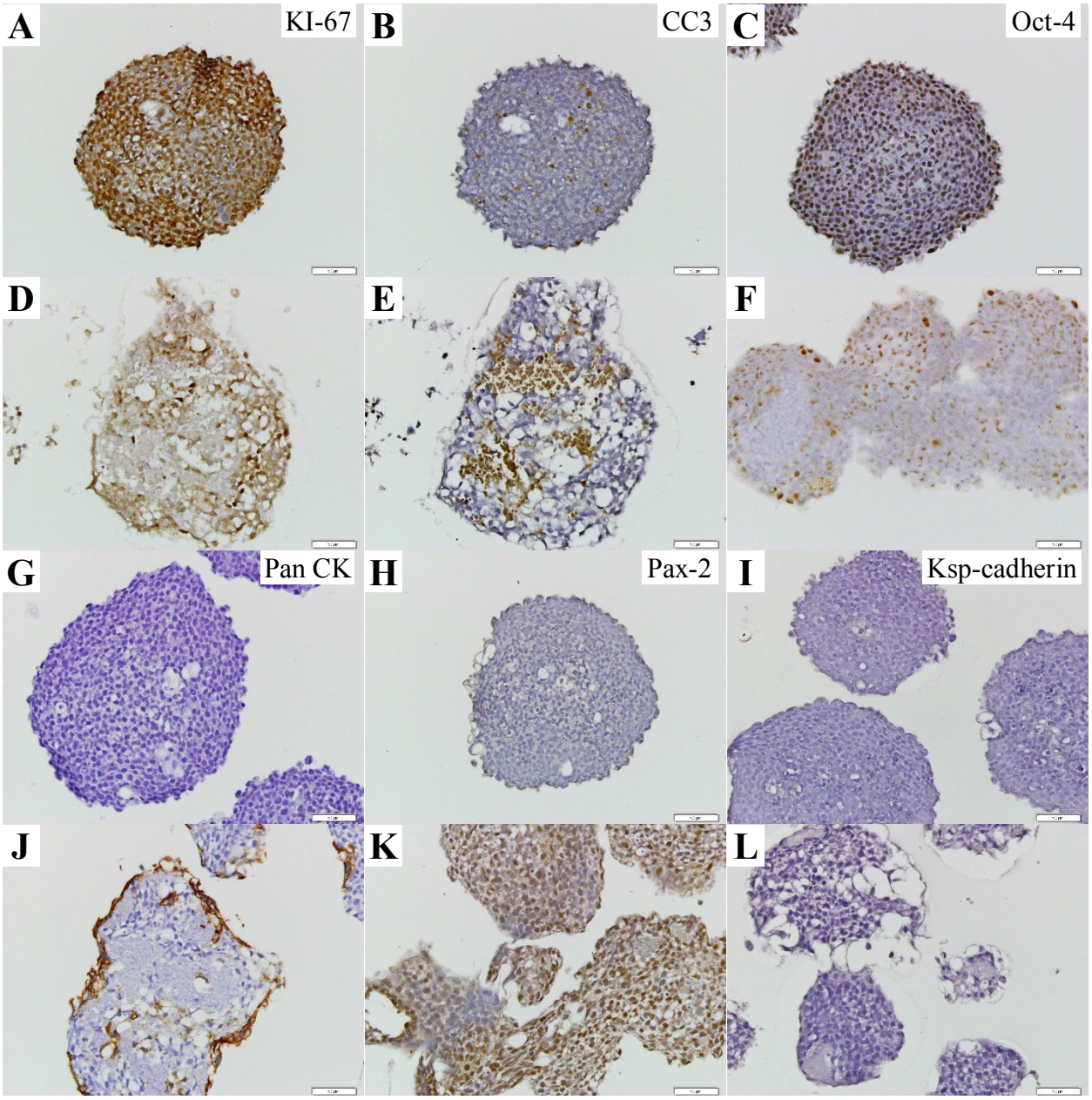

Figure B-3. Detection of key proteins for cell division, apoptosis, and differentiation in B5/EGFP ES cells cultured as embryoid bodies (40x magnification, bars denote 50µm). Ksp-cadherin IHC slides shown in vertical pairs, the top at day 0 and the bottom at day 10 or 14. A, D) KI-67 positivity shows most cells undergoing cell division at day 0 and many intact cells at day 14. B, E) CC3, a marker of apoptosis, is only present in a few scattered cells at day 0, but significantly larger areas at day 14. C, F) Partial retention of Oct-4 at day 10 shows remaining presence of undifferentiated stem cells. G, J) Pan-cytokeratin marks epithelial differentiation and is absent at day 0 and present at day 10. H, K) Pax-2 is present in intermediate mesoderm, ureteric bud, and cap mesenchyme populations and day 10 shows heavy positivity while day 0 is negative. I, L) Lack of KSP-cadherin at day 0 and 10 indicates no spontaneous differentiation into ureteric bud epithelium or polarized renal epithelium.

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BIOGRAPHICAL SKETCH

Matthew James Williams was raised in Michigan and graduated from Holland High School in 1993. He received a Bachelor of Science and Engineering in Mechanical Engineering from the University of Michigan in 1998. During his undergraduate years he was employed in car parts, furniture, and HVAC systems manufacturing. In his senior year, through a cooperative engineering program, he spent two semesters in automotive climate control test engineering at the DENSO International America Tech Center (Southfield, MI). He was also a member of the marching band and band service fraternity Kappa Kappa Psi. After graduation, he was hired as a project management consultant at ACM, Inc. (Ann Arbor, MI), and spent two years advising Ford Motor Company in program timing and resource management for EPA certification.

In 2000, in order to make a direct impact on healthcare, the author relocated to study biomedical engineering at the University of Florida. In 2005, he earned a master's degree in Biomedical Engineering for work in medical image processing under the guidance of Dr. Frank Bova. He programmed routines to improve segmentation of CT-based spine models for image guided surgery. Afterward, he transitioned to doctoral studies with a tissue engineering focus. Mentored by Dr. Chris Batich, his research tested the response of mouse embryonic stem cells to kidney extracellular matrix scaffolds for the purpose of organ generation. In 2009, he received an internship at RTI Biologics (Alachua, FL) for research and development of orthopedic implants. In 2011, the author spent a year as laboratory supervisor for Dr. Ed Botchwey at the University of Virginia. The Botchwey Lab pursued projects in regenerative medicine with focus on vascular remodeling, immune regulation, and endogenous stem cell recruitment. In 2013, the author completed his studies and was awarded a Ph.D. in Biomedical Engineering from the University of Florida. Matthew currently resides in St. Louis, MO, with his wife, Jennifer, and two children, Connor and Caden.