

(Re)Building a Kidney: moving toward new options for treating kidney disease

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Abstract

(Re)Building a Kidney is a National Institute of Diabetes and Digestive and Kidney Diseases-led consortium to optimize approaches for the isolation, expansion, and differentiation of appropriate kidney cell types and their integration into complex structures that replicate human kidney function. The ultimate goals of the consortium are twofold; to develop and implement strategies for *in vitro* engineering of replacement kidney tissue, and to devise strategies to stimulate regeneration of nephrons *in situ* to restore failing kidney function. Projects within the consortium will answer fundamental questions regarding human gene expression in the developing kidney, essential signaling cross-talk between distinct cell types of the developing kidney, how to derive the many cell types of the kidney through directed differentiation of human pluripotent stem cells, which bioengineering or scaffolding strategies have the most potential for kidney tissue formation, and basic parameters of the regenerative response to injury. As these projects progress, the consortium will incorporate systematic investigations in physiological function of *in vitro* and *in vivo* differentiated kidney tissue, strategies for engraftment in experimental animals, and development of therapeutic approaches to activate innate reparative responses.

Introduction

By current clinical definitions, approximately 10% of the adult population of the United States suffers from chronic kidney disease (CKD) ¹. Epidemiological studies suggest a similar incidence worldwide, and as populations age it is clear that finding new therapies for kidney disease is a global healthcare priority ¹. Kidney disease and injury impact diverse renal structures. Some, such as the proximal tubule, are regenerative and show highly proliferative responses to injury ^{2,3} while others, such as glomerular podocytes do not ⁴. Recent advances in our understanding of tissue regeneration, stem cell biology, and bioengineering make it feasible to develop new strategies for restoring kidney function.

The (Re)Building a Kidney Consortium

The (Re)Building a Kidney (RBK) consortium (www.rebuildingakidney.org) was formed by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) in 2015 to coordinate and integrate research on strategies to restore kidney function based on our understanding of kidney development, and the response of the adult organ to injury. The consortium focuses on two fundamentally different, but complementary strategies that have the potential to transform our approach to treating kidney disease and solving the kidney shortage crisis. The first is to engineer and engraft new kidney tissue. The second is to identify endogenous repair and regeneration processes in the injured kidney that can be exploited therapeutically. As depicted in Figure 1, the RBK aims to develop the tools, resources, and knowledge to: 1) characterize fetal and adult human kidney cell types to determine which cellular phenotypes should be targets for directed differentiation of human induced pluripotent stem cells (iPSCs), 2) produce and characterize human kidney progenitor cells from pluripotent cells while optimizing systems for their propagation, 3) generate complex and well-organized kidney tissue through controlled differentiation of natural and iPSC-derived kidney progenitor cells; 4) use biological scaffolds to promote tissue assembly and growth, 5) connect filtering nephrons to tubule networks to act as conduits for urine, 5) interrogate the potential of cell therapy to repair kidney damage, and 6) exploit endogenous repair mechanisms in adult human kidney to enhance regeneration.

Clues from kidney development: generating complex kidney structures from human iPSCs

Within the consortium, protocols based on recapitulating renal development have been adopted to coach human iPSC lines to form human kidney organoids ⁵⁻⁸. These organoids have been shown to contain all the anticipated components of the fetal human kidney, including patterned and segmented nephrons, collecting ducts, renal interstitium and endothelium (Figure 2) ⁸. As with protocols for the directed differentiation of iPSC to other human tissues (reviewed in ⁹), morphology and gene expression analysis suggests that kidney organoids represent first trimester human tissue⁸. The presence of derivatives of all major progenitors (cap mesenchyme, ureteric bud, vascular progenitors and stroma) suggests that these organoids could represent a promising source of human kidney tissue, although nephrons within the organoids are not fully differentiated, and lack functioning vasculature.

The ability to evaluate similarities between individual cell types present within iPSC-derived kidney organoids and the corresponding cells in human kidney is a high priority for the consortium. Although the transcriptional characterization of metanephric mouse kidney cells is well established, and the core pathways controlling nephrogenesis may be orthologously conserved, differences in gene expression presumably underlie important differences in renal architecture and function between mouse and human kidneys¹⁰. Thus, a key objective of the RBK is to generate a comprehensive gene expression database of the human kidney against which tissue derived from iPSCs can be compared. A rigorous microanatomical map will be established through RNA sequencing at the single cell level and exhaustive *in situ* hybridization analysis. New and powerful technologies for the capture of single cells are being used such as methods for analyzing RNA following intracellular sorting (MARIS), in which fixed cells are FACS-isolated for RNA-sequencing based on expression of intracellular antigens¹¹.

Achieving high-throughput efficiency in optimizing kidney organoid formation will require reliable and rapid means to detect the differentiation of different renal cell types. Currently there is a paucity of human iPSC lines expressing reporters of cellular differentiation suitable for the development of directed differentiation protocols. Capitalizing on knowledge gained from mouse and human kidney cell-type specific gene expression, fluorescently-tagged human iPSC reporter lines^{12, 13} are being generated using CRISPR/Cas9 gene editing approaches. These will allow both live imaging of *ex vivo* kidney differentiation as well as the isolation and transcriptional profiling of organoid-derived progenitors of the nephron, collecting duct and stromal lineages as well as differentiated podocytes, proximal tubules, and distal tubules. It is interesting to note that kidney organoids generated from human iPSC spontaneously form endothelial cell networks with accompanying perivascular cells⁸. While evidence exists for self-assembly of glomerular capillaries within some organoid glomeruli, the majority remain avascular⁸. Endothelial reporter iPSC lines are being generated to facilitate the isolation and characterization of this endothelium for comparison with the profiles of endogenous embryonic mouse kidney endothelium and human embryonic kidney tissue¹⁴.

Key concerns in developing a directed differentiation protocol are robustness and reproducibility; i.e. it is essential to determine a differentiation strategy that can be transferred from one stem cell line to another with reproducible outcomes in terms of organoid gene expression profiles and cellular complexity. With these concerns in mind, substantial focus is placed on sources of variation and the transferability of the approach between iPSC lines. Within the consortium a Quality-by-Design (QbD) strategy is employed to establish strict parameters for each component in the directed differentiation process^{15, 16}. This strategy allows for more than 12 dimensions within a design space in which morphogens and growth factors are tested simultaneously and in which interactions between individual signaling pathways can be determined. The end-point of the experiment is expression analysis of over fifty markers of renal cell types enabling evaluation of subtle differences between differentiation states. Robust differentiation protocols developed using this approach will be particularly important in disease modeling applications where uniform organoid composition is essential for reproducibility of high-throughput drug screening.

Understanding how human renal progenitor cells may be propagated in their undifferentiated state to numbers sufficient for trials of scaffolding and recellularization of organs is an important goal of the consortium. Based on *in vitro* mouse work that identified a cocktail of factors that mimic the *in vivo* renal progenitor cell niche¹⁷, efforts are currently focused on methods to culture and provide a source of phenotypically normal human nephron progenitor cells (NPCs) sufficient to generate synthetic kidney tissue scaled to the human. In addition, studies are being carried out to understand and mimic the extracellular environment of the natural NPC niche that confine highly motile NPCs within the cap mesenchyme and yet allow differentiation to all nephronal cell types within a synthetic tissue¹⁸. Directed differentiation of human iPSCs is of course another important source of human kidney progenitor cells. Efforts are underway to establish conditions for propagation of human cells at a variety of differentiation stages within the nephron lineage from organoids. iPSC lines that report on distinct differentiation states are being generated for these studies.

Putting the pieces together: scaffolding approaches for building human kidney tissue

Another priority within the RBK is exploring different approaches to generate synthetic kidney tissue that could then be transplanted into a host (Figure 3). In one approach, purified silk is being used to generate structures into which kidney cells derived from directed differentiation can be seeded and instructed to differentiate into mature kidney structures¹⁹. A second approach, also based on the use of polymers, involves 3D printing arrays of nephron tubules that can be layered to establish tissue. A third approach, is the recellularization of decellularized kidneys²⁰, for which transplant donor kidneys that do not fulfill quality control criteria constitute a plausible source. A fourth complementary approach aims to engineer extracellular matrix to maximize retention and presentation of relevant growth factors²¹.

Each of these approaches has distinct advantages. Scaffolds based on silk are extremely robust, and can easily be sterilized by autoclaving, modified with growth factors, and manipulated for engraftment¹⁹. Also, silk is in regular surgical use suggesting minimal hurdles for clinical application. Printing of nephrons has the advantage that structures can be easily organized in the stereotypical pattern seen *in vivo*, which is important for physiological function²². Adult decellularized organs provide the complex extracellular matrix environment required for attachment and function of differentiated kidney cell types^{20 23}. Engineering the matrix to present growth factors in a spatially organized fashion provides another approach to drive organoid differentiation on a larger scale²¹. The ultimate scaffolding solution may incorporate aspects of each of these bioengineering approaches. Further, it is important that the systems are also considered in the context of future needs *in vivo* to maximize translation potential for the work in the consortium.

Development of renal blood vessels is essential for the generation of functional nephrons, whether within bioengineered tissue or organoids.²⁴⁻²⁶ Recent results reveal that there is a surprising heterogeneity in endothelial cell gene expression within the developing kidney. How spatial and temporal differences in endothelial cell phenotype might impact nephron progenitor self-renewal or differentiation is an important and understudied area. Furthermore, re-generation of the nephron will require both pericytes and vascular smooth muscle cells for stabilizing capillaries and larger vessels, respectively. The consortium is developing

approaches for proper vascularization of organoids seeded in silk or other 3D scaffolds by 1) generating primary and conditionally immortalized Gli1+ mesenchymal stem cells (e.g., interstitial pericytes and perivascular fibroblasts), 2) developing methods for pericyte and vascular smooth muscle cell differentiation, 3) determining transcriptional profiles and molecular signatures of endothelial cell classes, and 4) identifying endothelial cell signals and crosstalk with interstitial and epithelial cells that impact kidney differentiation or proliferation within synthetic kidney tissue.

The patterning of the kidney stroma and interstitium is likely to be an essential event in the functional development of the nephrons. The renal interstitium arises along the cortical-medullary axis from two distinct progenitor populations marked by Foxd1 and Tbx18; the Tbx18 population gives rise to the interstitium surrounding the ureter, the renal pelvis and some of the papilla, while the Foxd1-positive progenitors give rise to the remaining interstitium including the pericytes, peritubular fibroblasts and mesangial cells^{27, 28}. Loss of the Foxd1-positive cell population affects the development of kidney tubules and vasculature non-autonomously¹⁸ and it seems likely that different interstitial zones could establish distinct molecular niches, impacting the final differentiation and function of kidney epithelia and endothelia. Also, much of the endocrine function of the kidney, including the production of renin and erythropoietin, is performed by interstitial cells that must be recapitulated in efforts to engineer or regenerate kidney tissue²⁹. With the goal of understanding interstitial cell differentiation, fluorescent reporter mouse strains are being generated for isolating interstitial cell types and techniques are being developed for co-culture with nephron and endothelial cells both in 2-D culture and in 3-D silk scaffolds. Transcriptional profiles derived from interstitial cell subpopulations should yield important insights into interstitial cell patterning and signaling roles.

To take full advantage of newly generated iPSC-derived replacement tissue to restore kidney function, filtering nephrons must connect to a collecting duct network to pass fluid. In kidney development, new nephrons derived from S-shaped bodies fuse lumens with the branched collecting system to generate functioning nephrons^{30, 31}. The signals guiding epithelial cells to invade an existing tubule, rearrange cell junctions, and form a new patent lumen are not known. The regenerating zebrafish kidney provides a quantitative model to test signaling pathways required for tubule invasion and nephron fusion. Following injury, the adult fish kidney regenerates by a stem cell mediated process, creating new nephrons which fuse to existing branched collecting ducts³². The consortium is taking genetic and pharmacological approaches to manipulating cell signaling during this process and translating new insights to how nephrons can be directed to "plumb into" existing collecting duct networks.

Enhancing endogenous repair

Understanding mechanisms of endogenous repair remains an important goal for regenerative nephrology. The consortium aims to understand cell-specific injury responses and the potential of adult progenitors to repair damaged kidney through regenerative processes. The proximal tubule epithelium has a robust capacity for repair after injury through the proliferative potential of surviving proximal tubule cells^{2, 3}. A goal of the consortium is to determine whether dedifferentiation confers unique functional properties or tubulogenic

potential on proximal tubule cells and to define the transcriptional profiles of these cells during the full course of repair. Cell types of particular interest include injured and dedifferentiated proximal tubule cells (defined by expression of *Havcr-1*)³³, interstitial pericytes and perivascular fibroblasts (defined by expression of *Gli1*)³⁴ and cells of the renin lineage^{35, 36}. These studies will also help inform efforts to generate high quality kidney organoids by providing transcriptional snapshots of injured versus healthy proximal tubule cells.

Adult podocytes are injured in many forms of glomerular diseases³⁷ and show very little, if any, ability to self-replicate⁴. However there are two podocyte cell progenitor populations under study in the consortium: the glomerular parietal epithelial cells^{4, 38, 39} and cells of the renin lineage^{35, 36}. Inducible reporter mice have enabled lineage tracing of both podocyte progenitor types under normal and experimental disease states. Isolation of each cell type is being performed to derive their transcriptional profiles with the goal of defining regulatory mechanisms controlling podocyte progenitor differentiation in health and disease. The cells will also be used to develop microfluidic "organs-on-chips", to provide efficient, reproducible, and cost effective multi-cellular cell culture models in native matrices⁴⁰. A goal of the consortium is to produce bioengineered 3D microphysiological flow directed systems^{40, 41} to recapitulate the glomerular filtration unit and provide a platform to examine replacement of depleted adult podocytes by local stem/progenitor cells: the glomerular parietal epithelial cells and cells of the renin lineage.

Consortium organization

The current composition of the consortium is described on the RBK website (www.rebuildingakidney.org). The consortium will continue to add new members and ideas through a Partnership Project Program administrated by the RBK coordinating center. Requests for applications in research areas that complement ongoing consortium studies will be announced annually. The coordinating center is a central data and resource repository for the consortium and the broader research community. Based on current consortium projects, the following types of data will provided. Gene expression: RNA-Seq data will be uploaded to the NCBI Gene Expression Omnibus (GEO) and links will be provided on the RBK website. In addition, the following will be archived and made publically available on the RBK website: Protocols: detailed protocols covering new methods developed in the RBK consortium. Examples include iPSC differentiation procedures, methods to generate biomaterials for use as scaffolds for kidney tissue, validation of qPCR for marker genes to identify key cell types and stages of differentiation. Reagents: databases for growth factor and small molecule activities and concentrations as well as a list of validated antibodies will be maintained. Images: Annotated micrographs, 3D representations of tissue structures, *in situ* hybridization data, and chemical structures. Reporter iPSC lines: Reporter lines are being generated from consortium-selected parent iPSC lines to enable the tagging of specific cell types for isolation and characterization. Once validated for pluripotency, genetic integrity and appropriate reporter gene expression, these will be listed on the website. Whole genome sequencing data of the parent iPSC lines will be available on the website as an aid to design efficient genome editing strategies.

RBK Consortium Trajectory: future challenges.

This review provides a snapshot of the consortium in its present form. As the individual projects within the consortium progress the focus of the program will evolve. Advances made during the initial phase will answer fundamental questions regarding human gene expression in the developing kidney, essential signaling cross-talk between distinct cell types of the developing kidney, how to derive the many cell types of the human kidney through directed differentiation, which scaffolding strategies have the most potential for kidney tissue formation *in vitro*, and basic parameters of the regenerative response to injury. In the next phase we anticipate focusing on physiological function of *in vitro* and *in vivo* differentiated kidney tissue, systematic investigation of strategies for engraftment in experimental animals, and development of therapeutic approaches to activate innate reparative responses (Figure 4).

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Figure 1

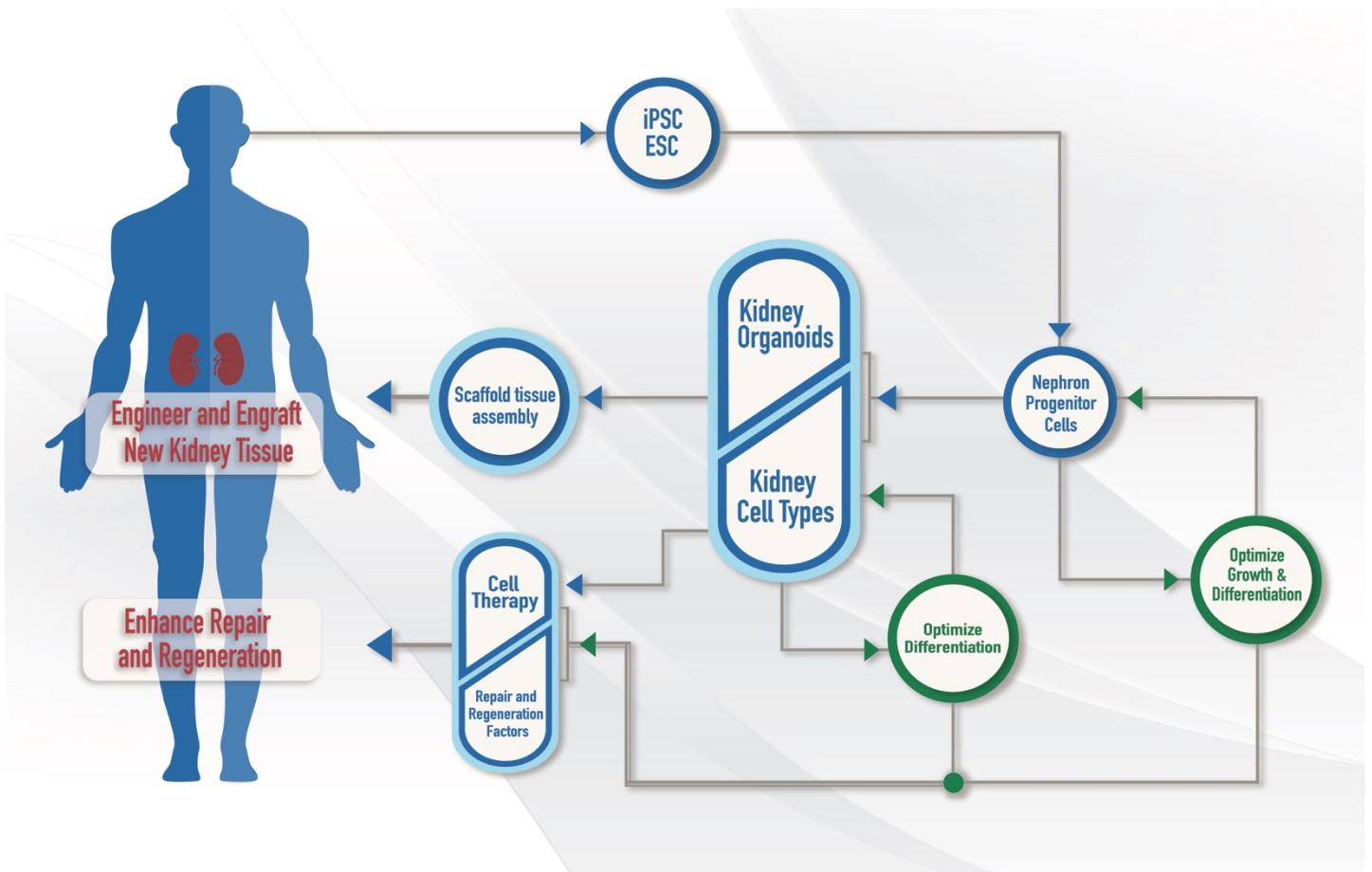


Figure 1. Goals of the RBK Consortium The ultimate goals of the RBK consortium are twofold: to engineer and engraft new kidney tissue and to enhance repair and regeneration of injured kidneys to restore function.

- **iPSC/ESC -> Nephron progenitor cells:** Propagating human progenitor cells in their undifferentiated state and using them to generate kidney cells and organoids, populate tissue scaffolds, and re-cellularize kidney matrix are key goals of the RBK consortium. Starting with induced pluripotent stem cells or established embryonic stem cell lines, nephron progenitor cells will be generated using differentiation protocols for nephrogenic mesoderm. Propagation and self-renewal of these cells and mouse cap mesenchyme nephron progenitor cells will be optimized using transgenic reporters of the progenitor cell type, qRT-PCR of qualified human marker genes, and single cell mRNA phenotyping as endpoints to detect expansion of the progenitor cell type.
- **Kidney organoids / Kidney cell types:** Differentiation of self-organizing organoids or directed differentiation of individual kidney cell types will also be achieved by optimizing medium conditions and assaying outcomes using cell-type specific fluorescent protein transgenic reporter iPSC / ESC lines, qRT-PCR of qualified human marker genes, and single cell mRNA phenotyping. Fluorescent protein reporter human iPSC lines will also allow live imaging of *ex vivo* kidney differentiation as well as re-isolation and transcriptional profiling of organoid-derived kidney cells including nephron and stromal progenitors, podocytes, proximal tubules, distal tubules and endothelium. Rigorously defined human kidney cell transcriptional signatures as well as cell injury markers derived from single cell RNA sequencing and MARIS will be essential for organoid and cell type quality control and to establish baseline phenotypes for further functional characterization, disease modeling and potential therapeutic use .
- **Scaffold tissue assembly:** Multiple approaches, including de-cellularized kidneys, silk and synthetic polymers, and 3D printed arrays of nephron tubules and blood vessels, will be tested to generate

structures into which differentiated kidney cells or organoids can be seeded, instructed to differentiate, and interconnect nephron tubules to form functioning kidney tissue with the potential to replace failed kidney function.

- **Cell Therapy / Repair and Regeneration Factors:** Defined growth factor activities optimized for progenitor cell self-renewal and organoid differentiation as well as for directed differentiation of renal cell types will be integrated into new approaches to promote endogenous repair of nephrons. Adult kidney podocyte and interstitial progenitor cells will be profiled and characterized to develop new ways to promote endogenous repair. Contributions of non-epithelial cells, the endothelium, interstitium, and pericytes/vascular smooth muscle, to kidney cell differentiation will also be assayed and modeled using fluorescent reporter lines and co-culture with nephron cells in 2-D and 3-D cultures.

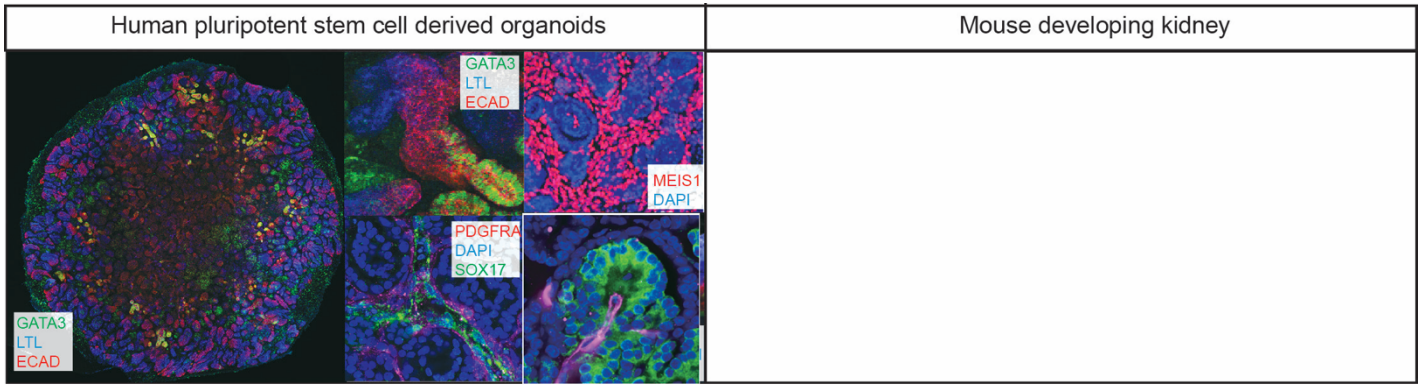


Figure 2. Comparison of human iPSC-derived organoids with fetal human kidney.

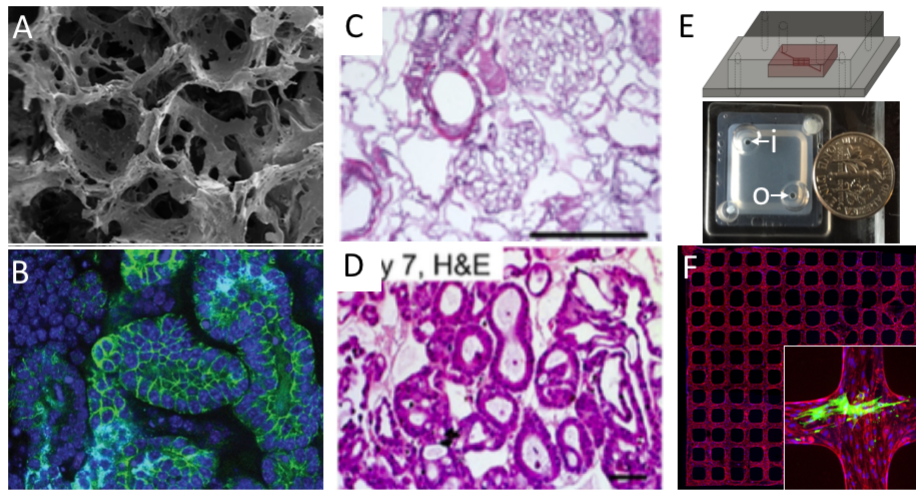


Figure 3. Engineering the extracellular environment. Several approaches to extracellular environment are being taken within the consortium. Polymer based (A, B), decellularized tissue (C, D), and organ-on-a-chip (E, F) approaches are shown. A. Scanning electron micrograph of 6% silk scaffold prior to cell seeding; a network of channels is formed into which nephron progenitor cells can be seeded. Photo: Jeannine Coburn. B. E-cadherin (epithelial cells) and DAPI (nuclei) staining reveals a network of tubules with lumens within the silk approximately 2 weeks after seeding nephron progenitor cells. Photo: Jessica Davis-Knowlton. C. Decellularized kidney tissue showing the extracellular matrix framework for tubules and glomeruli. Photo: Joseph Uzarski. D. Hematoxylin/eosin staining of a section of decellularized kidney that has been recellularized with MDCK cells 7 days after cell seeding. Photo: Joseph Uzarski. E. Schematic and top view picture of a vascular network-on-a-chip device that allows flow through the network via inlet (i) and outlet (o). Photo: ????. F. An example view of a 3D microvessel network formed by mouse kidney endothelial cells. Red: CD31, and blue: nuclei. The inset shows fluorescence immunostaining of a device in which podocytes (green) were cocultured with the vascular endothelial network (red). Photo: ???.

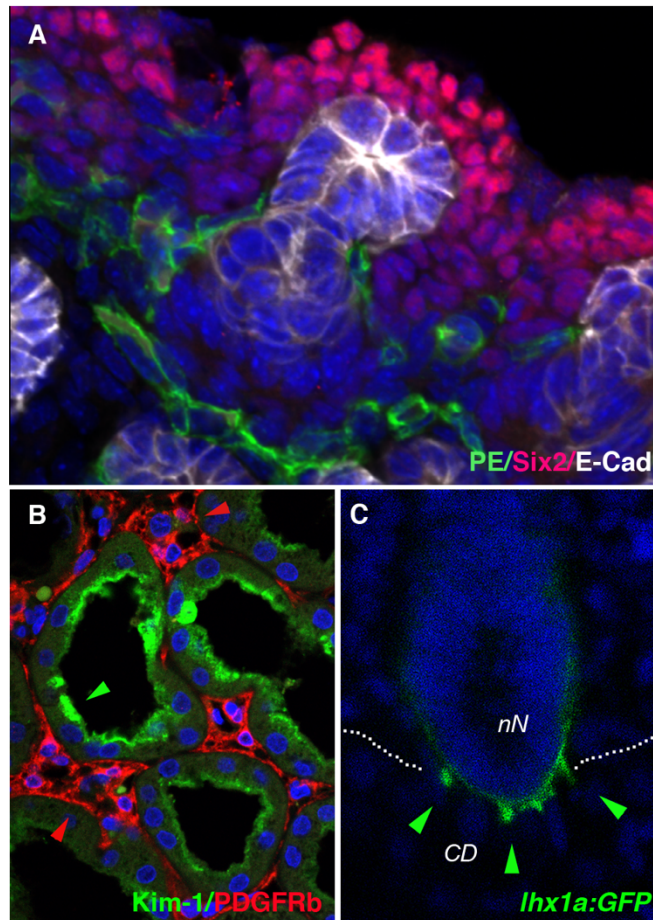


Figure 4. Challenges in building a kidney and promoting nephron repair. The kidney vasculature represents a variety of unexplored endothelial cell subtypes that may impact nephron differentiation through reciprocal signaling events. A) In the developing mouse kidney, vascular progenitor endothelial cells positive for Pecam/CD31 (green) are intimately associated with the nephrogenic cortical fetal kidney domain populated by Six-2 positive nephrogenic progenitor cells (red). E-cadherin (white) marks polarized epithelium of the branching ureter tips. (B) Kidney injury and fibrosis represents a major barrier to regeneration. Kidney injury recruits myofibroblasts, leading to fibrosis and chronic kidney disease. Kim-1 immunostaining (green) identifies injured and dedifferentiated human proximal tubule while PDGFRb immunostaining reveals activated adjacent interstitial kidney fibroblasts (red). Photo credit: Monica Chang-Panesso and Farid Kadyrov. (C) Nephron interconnection is an essential process in generating a filtering kidney. New insights from the regenerating zebrafish kidney, where many new nephrons are added to the existing collecting system, can help define signaling pathways that stimulate cell rearrangements that lead to interconnected tubule lumens. The *lhx1a:GFP* transgene (green) marks new nephron cell projections, or invadopodia (arrowheads), that penetrate the collecting system as a prelude to interconnection. CD: Collecting duct; nN: new nephron; blue fluorescence: DAPI nuclear stain.