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TITLE: INTRAVENOUS RENAL CELL TRANSPLANTATION FOR POLYCYSTIC KIDNEY DISEASE

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14. ABSTRACT Polycystic kidney disease (PKD), the most common life threatening genetic disease and affects approximately 1:400 people in the US including significant numbers of Active Duty Military personnel, Veterans and their beneficiaries. In PKD, the affected renal epithelia form cysts, eventually destroying renal architecture and function, leading to chronic kidney disease and end stage renal disease as well as pain, bleeding and infection of the cysts. Despite identification of the genetic mutations and remarkable advances in understanding the pathophysiology of PKD, there is currently no specific therapy for PKD. The present research examines the innovative translational therapy of intravenous administration of adult renal tubule cells containing the wild type (normal) PKD genes in experimental PKD (the PCK rat model). The aim is to replace abnormal renal epithelia while avoiding the morbidity and mortality of surgery. Expansion of cells in vitro will also extend the utility of organs available for transplant. Data obtained to date demonstrate markedly lower renal cyst volume and fibrosis and better kidney function with cell transplantation. These results are the basis for an additional funding application (PR130268) to extend the present studies, ultimately to prevent or treat kidney failure in humans. The results will also be presented at the Annual Meeting of the American Society of Nephrology in November, 2013.					
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INTRODUCTION: Polycystic kidney disease (PKD) is one of the most common genetic diseases. Because PKD occurs in up to 1:400 births, PKD is a common cause chronic kidney disease (CKD) and of end-stage renal disease (ESRD) in military, Veterans, their beneficiaries and the general population (1). Perhaps more importantly, the transplantation techniques developed in these studies will be relevant to acute and chronic kidney disease of other etiologies which disproportionately affect Veterans.

BODY: Despite identification of the genetic abnormalities leading to PKD, and huge advances in understanding the pathophysiology of PKD, there is no effective therapy to prevent or limit progression of renal dysfunction to ESRD in PKD. The number of PKD ESRD patients may be increasing: USRDS 2013 (1) reports 28.2 (per million population) PKD patients on dialysis in 1985, 62.9 in 2000 and 92.5 in 2011. Although these data may reflect better diagnosis and reporting, they illustrate that ESRD from PKD is a huge health problem. The main goal of this proposal is the development of effective cell therapy protocols that prevent disease progression in PKD. A secondary aim is to understand the mechanisms of renal healing by cell transplantation, including reduction in cyst volume and renal scar formation. The results may also positively affect other forms of chronic kidney disease (CKD) and ischemic injury. The ultimate goal is prevention of ESRD in Veterans and others with kidney disease.

Dr. Dominguez and his team have developed, with current funding from the DoD and VA, novel cell transplant protocols that improve renal function and structure in multiple models of renal failure (2-5). The overall experimental design is presented in figure 1. Adult tubular cells are harvested from normal male SD rats. The kidneys are removed and tubules immediately separated by collagenase digestion and cultured in epithelial medium (4-5).

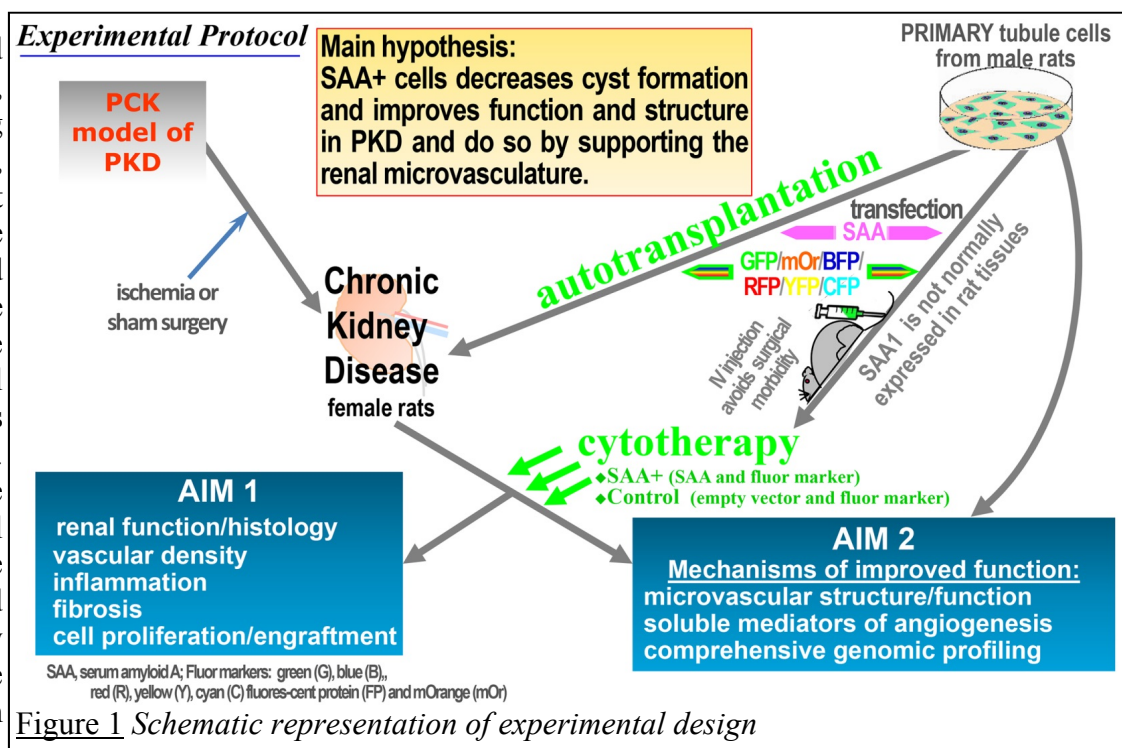


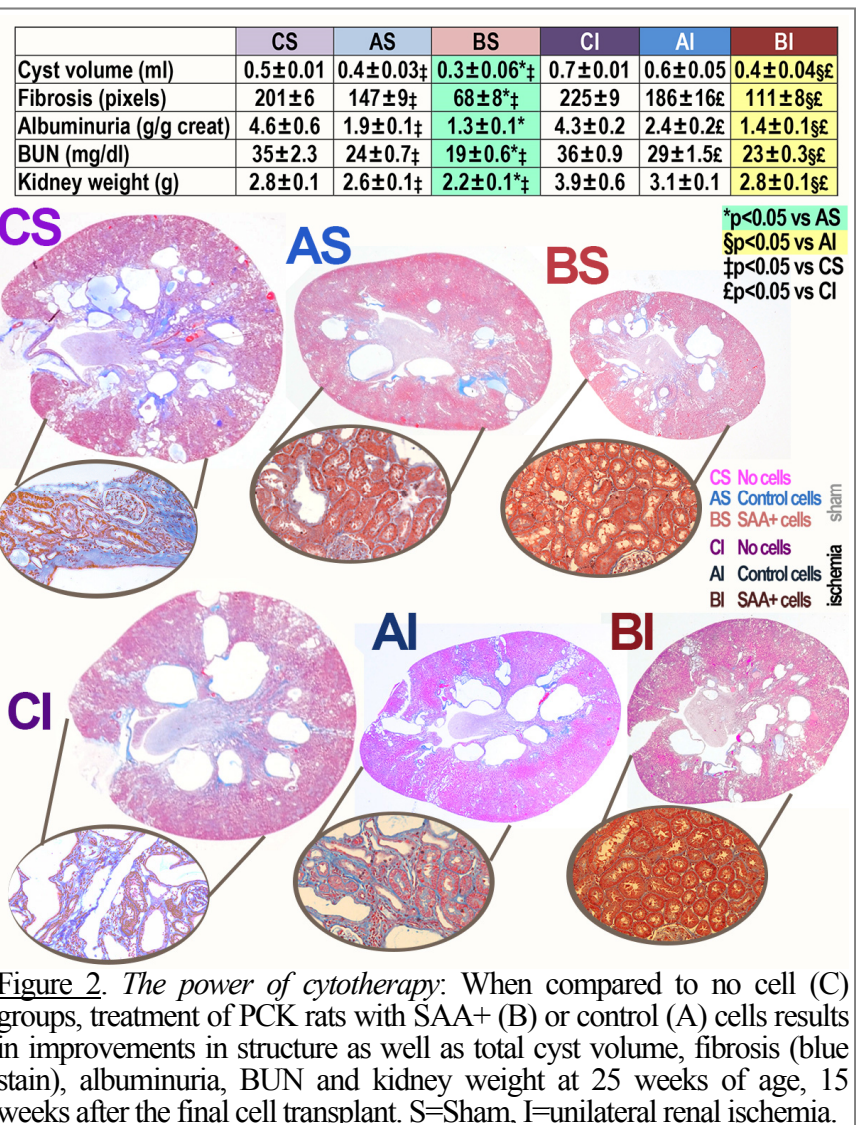
Figure 1 Schematic representation of experimental design

The isolated tubules are then transfected with plasmid constructs designed to over-express the gene for Serum Amylase A1 protein (SAA) and also for green fluorescence protein (GFP). The latter serves to track the cells in vivo after the transplants. SAA is an acute phase reaction protein added for two main reasons, SAA induces a strong tubulogenic program in cultured renal epithelial cells and it also enhances their proliferation. We typically get much better results when the transplanted primary renal cells express SAA (4-5). After one week in vitro the cells were ready for transplantation via the tail vein of female rats; typically 2×10^6 cells/rat.

PRELIMINARY DATA: CKD has reached epidemic proportions and results in immense suffering and loss of life, so the introduction of novel and effective therapies is urgently needed. We found that non-invasive renal cell transplantation improved survival and kidney function in diverse models of renal

failure in rats (3-5). The transplant protocols have the advantage of using cells from one kidney to *rescue function in multiple* animals and are based in these preliminary observations:

- ♦ The acute phase protein serum amyloid A (SAA) is critical in tubule formation (Kelly KJ, Kluge-Beckerman B and Dominguez JH, Am J Physiol 296: F1355, 2009)
- ♦ Non-invasive infusion of SAA expressing renal tubule cells significantly improves function and structure in established renal failure in multiple models (Kelly KJ et al, Am J Physiol 299: F453, 2010 and Kelly KJ et al, Am J Physiol 303: F35, 2012), including: Acute kidney injury (AKI) due to ischemia, gentamicin or cisplatin-mediated injury. Chronic kidney disease secondary to diabetes, polycystic kidney disease or cisplatin. Auto-transplantation of cells harvested from an injured kidney rescues function in the *same animal* with CKD due to cisplatin (4). From this preparative work we documented that unlike stem cell protocols, male donor SAA expressing cells labeled with green fluorescent protein (GFP) can be demonstrated in female recipient kidneys months after cell transplantation. We have found that direct exposure of cultured renal tubular cells to SAA results in formation of functional tubules that transport the organic anion fluorescein and express characteristic tubule transporters (3,4). SAA is also expressed during tubule formation *in vivo*, in the mouse embryo and during renal regeneration after AKI, consistent with its critical role in tubulogenesis (3,4).



KEY RESEARCH ACCOMPLISHMENTS:

- To assess the ability of SAA expressing cells to support reconstitution of tubules *in vivo*, rats were given gentamicin, cisplatin or subjected to renal ischemia. After renal failure was established, 10⁶ control (transfected with empty vector as well as green fluorescent protein, GFP) or SAA expressing (transfected with SAA and GFP) renal tubular cells were injected intravenously. **Mean serum creatinine was significantly better after treatment with SAA+ cells in each model** and infused GFP+ cells were found in recipient kidney tubules 1, 5 and 7 days after injection (4,5).
- These studies were then extended to primary cells to foster clinical applicability. Primary renal tubule cells transfected with SAA also rescue function in established AKI (4,5). Significantly better survival and histology was also seen in the groups that received SAA+ cells (4,5). **Treatment with SAA expressing cells also improves renal function and structure in other models of renal failure:** CKD due to cisplatin-mediated injury (4), diabetic nephropathy (Am J Physiol. Renal in press) and in PKD (figure 1).

- The current report is dedicated to research on the PCK rat model of adult recessive PKD (6). The PCK rat model has significant advantages: The mutated gene in the PCK rat (PKHD1 or fibrocystin) is orthologous to the human gene; the phenotype is very similar to the human phenotype in both autosomal dominant (ADPKD) and recessive (ARPKD) PKD. Furthermore, cystic disease in the

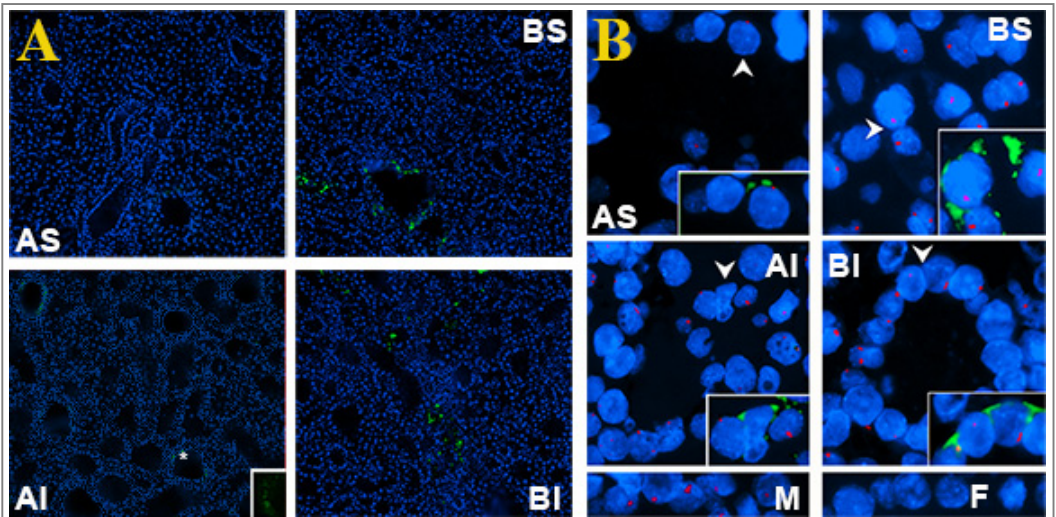


Figure 3. Engraftment of transplanted cells: PCK kidney sections at 25 wk of age show GFP+ cells in tubules and cysts (A). In AI, green fluorescence of the area at the asterisk is shown in the inset. Engraftment is also apparent as Y chromosome+ (red) in renal nuclei of females that received male cells (B). The insets show green fluorescence of areas at arrows, showing GFP and Y chromosome in the same cells. Nuclei are stained with DAPI (blue). AS, control cells/sham surgery; AI, control cells/ischemia; BS, SAA+ cells/sham; BI, SAA+ cells/ischemia; M, normal male; F, normal female.

PCK rat, while extensive, allows time to intervene, whereas in other models, cystic disease progresses too fast for successful intervention and, in that respect, those super aggressive models differ from human PKD.

- The PCK rats were intervened at 8 weeks of age, one group was sham operated and the other was subjected to unilateral renal ischemia for 50 minutes. The purpose of the surgery was to eliminate as many genetically defective tubular cells –carry the mutated PKHD1 gene– as possible and make room for normal tubular cells. The rats were then transplanted, receiving cell infusions, either SAA- (A) or SAA + (B) cells, 2 days, 2 weeks and 4 weeks after surgery. The renal tubular cells were obtained from normal SD male rat donors, selected because of their related genetic background. The rats were terminated at age 25 weeks of age, or 13 weeks after the last cell transplant. There were two additional rat control groups that did not receive cell transplants. In summary the six PCK rat groups were:

1. Sham operated rats receiving SAA- (AS) cells (n= 5)
2. Sham operated rats receiving SAA+ (BS) cells (n =5)
3. Unilateral renal ischemia rats receiving SAA- (AI) cells (n = 5)

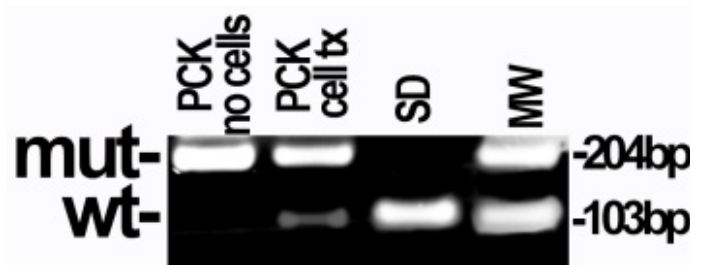


Figure 4. Genotype of the PCK mutation: PCR demonstrates the presence of both wild type (wt) and mutated (mut) PKHD1 genes in kidneys from rats that received SAA+ cells but not in untreated PCK or normal Sprague Dawley (SD) rats. PCR genotype was performed using primers specified by Charles River:

Mut-F: 5'-AAG CCA AAT CTT TCT CTT TTC CT-3'
 Mut-R: 5'- CTT GCT GTC CGA ATA CCA C -3'
 Wt-F: 5'-ACT GCC TTT TAC TGA AGC ATT TAA C-3'
 Wt-R: 5'- TGG AAG GAA AAG TTG CCC T -3'

MW, molecular weight markers

4. Unilateral renal ischemia rats receiving SAA+ (BI) cells (n = 5)
5. Control sham operated rats, No cells (n = 5)
6. Unilateral renal ischemia rats, No cells (n = 5)

Although the mechanism of renal failure in PKD is distinct from other forms of CKD, we hypothesized that cytotherapy with renal cells from male SD rats containing the normal PKHD1 gene would improve structure and decrease cystogenesis in PKD. The rats survived with the exception of two rats in the AI group, which died two weeks after the second infusion. The remaining PCK rats were terminated at 25 weeks of age (15 weeks after the final cell dose), **total cyst volume, renal fibrosis (quantified on trichrome stained sections), albuminuria, blood urea nitrogen (BUN) and kidney weight were significantly decreased in groups treated with SAA+ cells as compared to those groups that received SAA-control cells (figure 2).**

Unlike stem cell protocols, infused cells are

found in kidneys weeks after the last cell transplant (our Ref 4, 5 and also figure 3). In addition to identifying the GFP+ cells, continuous engraftment is documented using fluorescence in situ hybridization (FISH) for the Y chromosome (red) in

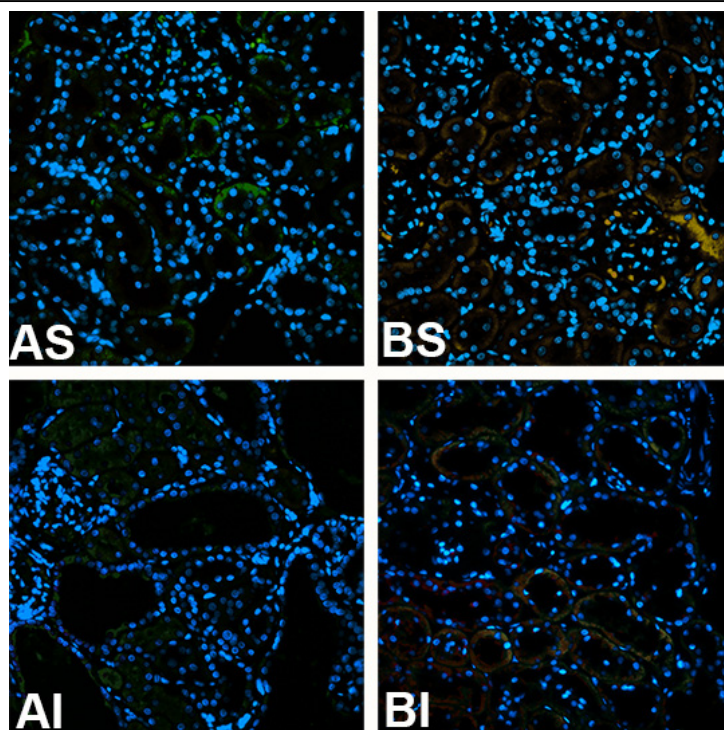


Figure 5. Expression of SAA1 co-localizes with GFP in PCK rats given SAA+ cells: Representative images from kidneys stained for SAA (red) show GFP+ cells in kidneys from rats that received control (A) cells. SAA co-localizes with GFP (resulting in orange) in the kidneys from the groups that received SAA+ cells. All nuclei are labeled with DAPI (blue). AS, control cells/sham surgery; AI, control cells/ischemia; BS, SAA+ cells/sham surgery; BI, SAA+ cells/ischemia

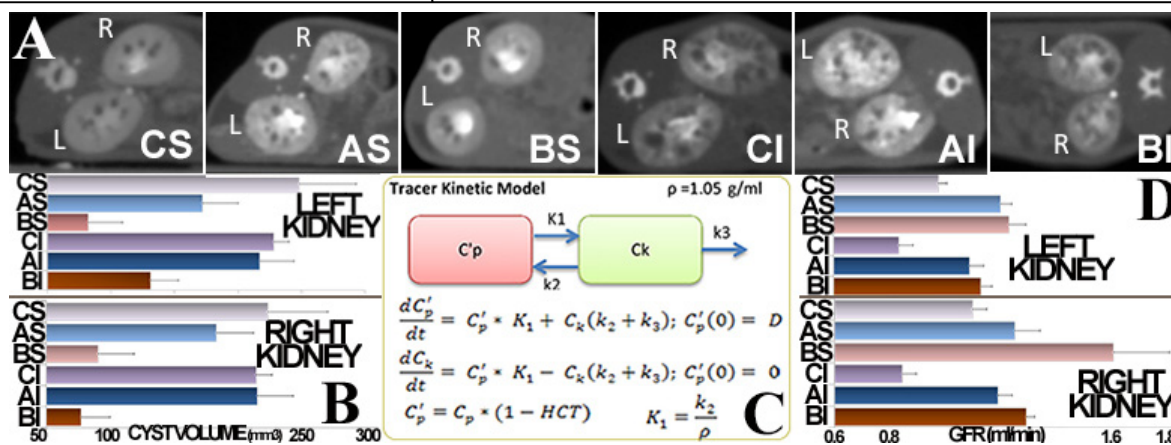


Figure 6. Dynamic contrast computed tomography imaging of decreased cyst volume with cytotherapy: Representative CT images of kidneys from 25 wk old PCK rats treated with control cells, SAA+ cells or no cells following sham surgery or unilateral renal ischemia (A). Graphs of preliminary data in 17 rats show improvement in cyst volume (B) and GFR (D) with SAA+ cells. In addition, control cells result in improvement when compared to the “no cell” groups. The 2 pool model for determination of GFR is shown in C. C, concentration; p, plasma; k, kidney; K, constant; t, time; hct, hematocrit; ρ , density of contrast media (1.05g/ml); CS, no cells/sham surgery; CI, no cells/ischemia; AS, control cells/sham surgery; AI, control cells/ischemia; BS, SAA+ cells/sham surgery; BI, SAA+ cells/ischemia

kidneys from female rats that received male cells (figure 3). Moreover, PCR shows both the normal and mutated PKHD1 gene (figure 4) and the presence of the male determining SRY gene in kidneys from rats that received SAA+ cells (not shown). SAA protein can also be demonstrated in groups that received SAA+ cells, figure 5.

To determine the tubule segments from which cysts originate, kidneys from PCK rats were labeled with lectins *dolichos biflorus* (conjugated to rhodamine) and *lotus tetragonolus* (pseudo-colored cyan) to label collecting ducts and proximal tubules, respectively. Cystic dilation was seen largely in collecting ducts with some cysts emerging from proximal and unlabeled tubules. GFP+ (transplanted) cells were found in both proximal and collecting duct cysts (not shown).

We have also examined the severity of cystic disease and split renal function by dynamic CT (figure 6). In preliminary results obtained 2 weeks before termination, total cyst volume was less and renal function (glomerular filtration rate, GFR) better in rats treated with SAA+ cells.

- HYPOTHESIS:** Data from others and our own data (from work conducted for the Department of Defense, Discovery Award PR110473) guided us to propose the following testable hypothesis: “**intravenous transplantation of rat adult kidney cells, expanded and re-programmed in culture, limits cyst formation and improves structure and function in polycystic kidney disease**”. Accordingly, the implicit mechanisms of cell action are based on limiting cyst growth. The assumption is that cyst growth is a critical determinant of progression at two fundamental levels: 1) Cyst expansion compresses and damages surrounding functional tissue rendering it dysfunctional. In figure 7 we illustrate this point showing the compressing effect of an expanding cyst on a glomerulus in its vicinity. 2) Cyst expansion demands dedicated blood supply, recruiting enough blood vessels to form dense vascular capsules around the emerging cysts, figure 8. One consequence of the vascular sequestration is a limited and sparse peritubular vasculature in functional tubules. We presume that these thin vascular networks are inadequate and enhance tubular susceptibility to ischemic injury, a pro-cystogenic stimulus.

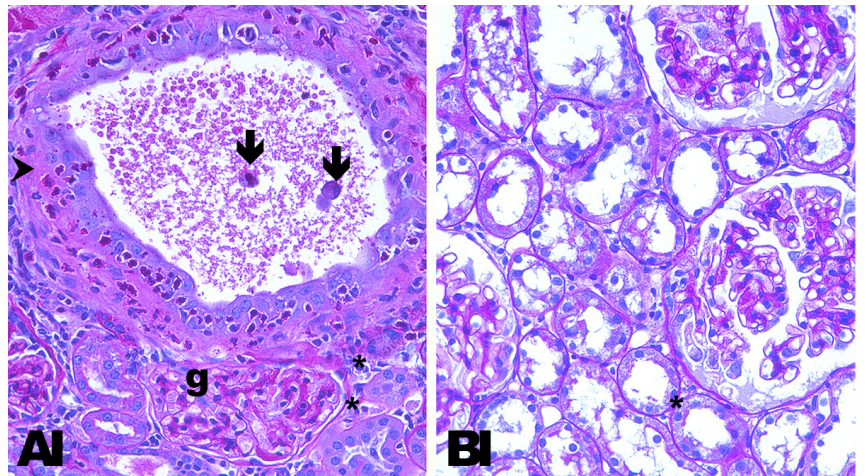


Figure 7. Post-ischemia PCK kidneys: Representative PAS stained kidney sections from 25 wk old PCK rats post-ischemia at 6 wk of age and cell transplanted three times with SAA negative cells (AI) or SAA+ cells (BI) are shown. In AI, the expanding renal cyst contains leukocytes (arrows) and crushes a glomerulus (g). The arrow head points to the fibrotic cyst capsule. The asterisks are in peritubular spaces filled with leukocytes. BI only shows microcysts, glomerular structure is grossly preserved, and peritubular spaces are devoid of inflammatory cells.

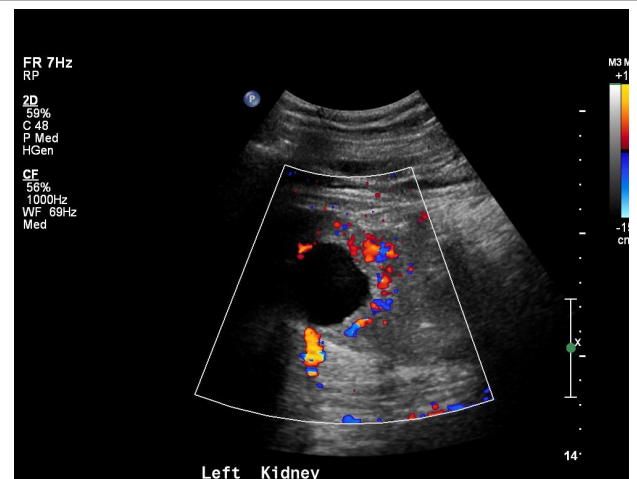


Figure 8. Renal Doppler/US: Vascular capsule around a cyst in human PKD.

- **FUTURE WORK:** Our future work will be focused on the role of the cell transplants on the microvasculature in the PCK rat. We anticipate that the transplanted normal cells will mainly reside in developing micro cysts and limit their expansion by correcting the polarity of adjacent epithelial cells. The new hybrid tubules may recruit more blood vessels and improve delivery of oxygen and nutrients.

REPORTABLE OUTCOMES: American Society of Nephrology annual meeting invited presentation November 2013.

Additional DOD research funding application submitted to extend these studies, ultimately to humans (Application # PR130268).

CONCLUSION: polycystic kidney disease and the resultant renal failure result in tremendous suffering and loss of life in Veterans and the general population and the novel, non-invasive cytotherapy protocols reported here have tremendous potential to help large numbers of patients with CKD and ESRD. Our renal regeneration protocols also have the potential to limit the progression of CKD and even restore function when patients reach the point of ESRD.

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APPENDICES: Abstract for presentation to be delivered at the Annual Meeting of the American Society of Nephrology in November, 2013.

SUPPORTING DATA: Figures embedded in the main text.

APPENDIX

Abstract to be presented to the Annual Meeting of the American Society of Nephrology, November 8, 2013.

Intravenous renal cell therapy for autosomal recessive polycystic kidney disease in rats

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Polycystic kidney disease (PKD, including ADPKD1, ADPKD2, ARPKD) is a group of diseases with overlapping phenotypes and are not curable. They are enhanced by renal injury which accelerates progression to ESRD, and then the only hope to get off of dialysis is kidney transplantation, but the need for organs to transplant exceeds supply. We have introduced intravenous renal cell transplantation (IRCT) in rats as a future alternative to kidney transplantation, and tested the hypothesis that IRCT with Serum Amyloid A protein (SAA) expressing normal adult renal cells improves structure and function in PKD by coordinating the re-orientation of adjacent host PKD cells. We tested this idea in the PCK rat, an orthologous model of ARPKD. We included four rat groups starting with surgery at 6 weeks of age and IRCT at 6, 8, and 10 weeks of age, termination was at 26 weeks of age: SA, were sham operated and given SAA negative cells, n=7. SB, were sham operated and given SAA positive cells, n=7. IA, had unilateral renal ischemia for 50 min and given SAA negative cells, n=8. IB, had unilateral renal ischemia for 50 min and given SAA positive cells, n=8. We followed renal function and structure with Dynamic Contrast Enhanced CT (DCE-CT). Donor cells were found in abundant numbers on the cyst walls and damaged tubules, but not outside the kidneys. The SAA positive "B" cells improved function and structure as detailed in table:

	AS	BS	AI	BI
Kidney Weight (g)	2.60±0.19	2.18±0.39	3.11±0.16	2.80±0.33
Albuminuria(g/g creat)	1.93±0.14	1.26±0.11	2.40±0.16	1.42±0.13
BUN (mg/dl)	24±0.6	19±0.6	29±1.5	23±0.3
Cyst Volume(ml)	0.43±0.02	0.26±0.07	0.57±0.11	0.36±0.02
GFR (ml/min)	1.08±0.05	1.28±0.08	0.097±0.09	1.32±0.09

IRCT is an effective means to retard cyst development and progression (all "B" groups significantly different than corresponding "A" groups, $p < 0.05$) in the PCK and has a great potential for PKD therapy.

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