Visualization of Kidney Dynamics

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Functional and Therapeutic Studies *in Vivo*

- **Intact Kidney**
  - Tubule
  - Cell
  - Hormonal
  - Vasculature

- **Isolated Perfused Kidney**
  - Tubule
  - Cell

- **Isolated Perfused Tubule**
  - Tubule
  - Cell

- **Isolated Cells**
  - Cell

*Multi-photon microscopy*

Molitoris and Sandoval AJP, 2005
Visualizing Glomerular Function
500K Mw FITC-Dextran (Green)

10K Mw Rhodamine Dextran (Red)

Hoechst 44432 (Blue)
Glomerular Permeability and Vascular Clearance
Reducing Scan Size

5 Frames/sec
500kDa FITC Dextran with 3kDa TR Dextran Injection
Visualizing Filtration the Basement Membrane & Podocyte

- Normal Glomerular Capillary:
  - lumen
  - endothelial cell
  - mesangial cell
  - mesangial matrix
  - epithelial foot process
  - basement membrane

- Diagram showing filtration slit (40 nm), slit diaphragm, epithelial podocytes, glycocalyx, and fenestrae.
Question:
What are the Underlying Mechanisms of Proteinuria?

Hypothesis:
Glomerular Filtration as well as Proximal Tubule Reabsorption are Critical Determinants of Proteinuria

**Current model**
- GSC < 0.001
- Apical uptake by receptor-mediated endocytosis
- Delivery of degradation products
- Excreted amount << Filtered amount

**Proposed model**
- GSC ~ 0.04
- Apical uptake by receptor-mediated endocytosis
- Intact delivery by transcytosis
- Excreted amount << Filtered amount

**Figure 1** Comparison of the current model of renal albumin handling, as described in most of the publication, with the new model proposed by Russo et al. GSC, glomerular sieving coefficient.
Rf-2 Gene Modulates Proteinuria and Albuminuria Independently of Changes in Glomerular Permeability in the Fawn-Hooded Hypertensive Rat

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We report that Rf-2, a gene within the Rf-2 locus, appears to influence the development of proteinuria (UPV) and albuminuria (UAU) in fawn-hooded hypertensive rats (FH/H). Using congenic animals, we narrowed the region to eight genes, however, only one gene had a sequence variant. Rf/H had a mutation in the start codon, resulting in a natural knockout in the FH/H strain. Despite no differences in glomerular albumin permeability, congenic animals carrying the wildtype Brown Norway (BN) allele of Rf/H on the FH/H background exhibited, on average, 40% and 60% less UPV and UAU, respectively, than FH/H. These findings suggest that Rf/H may modulate the tubular processing of filtered proteins without affecting the glomerular filtration barrier. This is the first report for an animal model of hypertension-associated renal failure. The gene resides on human chromosome 11, which has been linked to renal disease.

The genetic dissection of quantitative traits, such as renal failure, has proven a challenging task in humans because of their polygenic nature and interactions with the environment (9,27). One solution is to use animal models to study the genetic basis of disease. The first direct genetic evidence for hypertension-associated renal disease came from the FH/H strain, in which five genetic regions or quantitative trait loci (QTLs) (RF through R3) have been linked to the development of UPV, UAU, and focal glomerulosclerosis (23,24). Since then, several groups have found the homologous regions in humans to be also linked to renal failure (10,33,37). The Rf-3 locus, located on rat chromosome 11, showed a recessive mode of inheritance with significant linkage to UPV (logarithm of the odds ratio score 5.39) and UAU (logarithm of the odds ratio score 6.59) (1). This locus accounts for approximately 30 to 40% of the total protein excretion (1). Studies to other rat models of renal failure have also implicated the Rf-2 region in the development of UPV and UAU (35,28,36,29). In addition, Wang et al. (67) have reported linkage to a familial form of focal segmental glomerulosclerosis (FSGS) in a region of human chromosome 11 that overlaps with the Rf-2 locus. In this study, we report that a natural knockout of the Rf-2 gene, likely the Rf-2 gene, is associated with the Rf-2 gene expression in UPV, UAU, and the permeability of isolated glomeruli to albumin. Finally, through comparative genomics, we constructed a map of the synteny between the rat and human QTLs at the gene level of resolution.

Materials and Methods

Generation of Congenic Animals and Sequencing of Candidate Genes

All experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Congenic animals were developed by marker assisted selective breeding of FH/H and BN rats as reported previously (25). Sequencing of potential candidate genes was performed using genomic DNA and cDNA on an ABI3700 capillary sequencer according to the manufacturer's suggested protocol.

Urinary Protein/Albumin Excretion and Assessment of Glomerular Permeability

Using 12-week-old animals, fed standard rat chow, was collected over 24 h periods and analyzed for total protein by the Wellcome-Bolin-De inspiring method (56). Albumin excretion was assessed using the AB509 assay (46). Results are reported as the average of the two consecutive days.

Glomerular permeability was determined using an in vitro functional assay as described previously (68).

Blood Pressure Measurement

BP was measured directly, in conscious rats, by correlation of the right femoral artery as reported earlier (24).

Western Immunoblotting

Proteins from 100 mg kidney homogenates of 12-week-old FH/H, BN, and FH/H.Rb38 con-
FIG. 2. Glomerular filtration rate in fed and fasted rats. Lines join data from same animals.
FIG. 1. Daily proteinuria in fed and fasted rats. Lines join data from same animals.
Albumin Filtration and Reabsorption in the Rat
Albumin Filtration and Reabsorption in the Rat
Effect of Early Diabetes in the Rat on Albumin Filtration and Reabsorption

69 kDa FITC -Dextran

ALEXA 586 Albumin

Russo et.al. JASN 2009
Effect of Early Diabetes in the Rat on Albumin Filtration and Reabsorption

Russo et al. JASN 2009
Challenges

1. Dogma, Assumptions, Biology, Reagents, Sensitivity
2. Quantitative Analysis without Gold Standards
3. You See What you are Looking For
4. Correcting for Depth of Field
5. Going Deeper
6. Out of Focus Fluorescence
7. Physiologic state of the rat
24 Hr CLP Glomerular Flow Heterogeneity

Large 150 kDa dextran

Small 3 kDa dextran
Quantifying Glomerular Filtration
GFR as a Marker of Kidney Function in AKI

1. Historical Marker of Global Kidney Function
2. Multiple Techniques but either Lack Accuracy or Speed of Determination
3. No Clinically Usable Technique for AKI
4. In AKI would have Diagnostic and Severity of Injury Capabilities
5. An Accurate GFR would allow for Earlier Initiation and Termination of RRT
6. May have a Role in Surveillance Technology
Ideal Characteristics of GFR Technique

1. Safe, Inexpensive, Repeatable and Accurate
2. Rapid at Bedside Readout
3. Display Data for Interpretation and Evaluation of Test
4. Minimally Invasive or Noninvasive
5. Administered by Nursing Personnel
6. Independent of Vascular Permeability
Why is GFR not Determined in AKI Now?

1. Multiple “Gold Standard” Techniques have been developed

2. Sampling methods such as inulin, iohexol, or iothalimate clearance:

   • All require 6+ hours to administer test - multiple blood draws
   • Require samples to be sent for outside lab analysis, requiring days
   • Possible radiation exposure from injected marker
   • Require moving the patient
   • Too expensive, time consuming and cumbersome to be practical
SCr and eGFR: Inadequate Measures in AKI

- By the time SCr rises above normal, 50% of kidney function has been lost
Quantifying Glomerular Filtration
Quantifying Glomerular Filtration in Rats

Data Collection Only Required 10-15 minutes per GFR Determination Post Injection

Advantages of Dextran
- Solubility
- None Immunogenic
- High labeling efficiency

Converting from a Microscope to Portable Technique

1. Fluorescent Markers, No Change form Microscopic Technique
   A. Large Dextran for Vascular Volume Measurement
   B. Small Free Filterable Dextran for Rate Determination

2. Excitation Generation and Emission Detection Device Needed

3. Optical Probe for Delivery of Excitation Pulse and Recovery of Emission Signal

4. Data Storage and Software Analysis

5. Raising MONEY
“Advancing” to the Dark Side

Academia → Industry
Commercialization: Bench to Bedside

Discovery using expensive 2-photon laser requiring surgery to visualize exteriorized kidney

Inexpensive, portable LED-based device using fiber optic introduced through standard 18g catheter to read markers
Measuring Fluorescence Through 18g Catheter
FAST Clinical GFR Technique
GFR Determination in Dogs Via A Peripheral Vein
New Technology In Development for Rapid GFR

1. Repeatable, accurate, rapid GFR determination at bedside
2. Minimally invasive optical device
3. Rich data for interpretation and evaluation of test
4. Able to be administered by nursing personnel
5. Independent of vascular permeability
**Table 1. Investigational uses for multi-photon microscopy**

<table>
<thead>
<tr>
<th>Glomerular</th>
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</thead>
<tbody>
<tr>
<td>Size/volume</td>
</tr>
<tr>
<td>Permeability/filtration</td>
</tr>
<tr>
<td>Fibrosis/sclerosis</td>
</tr>
<tr>
<td>Microvasculature</td>
</tr>
<tr>
<td>RBC flow rate</td>
</tr>
<tr>
<td>Endothelial permeability</td>
</tr>
<tr>
<td>WBC adherence/rolling</td>
</tr>
<tr>
<td>Vascular diameter</td>
</tr>
<tr>
<td>Cellular uptake</td>
</tr>
<tr>
<td>Cell type-specific uptake</td>
</tr>
<tr>
<td>Site – apical vs. basolateral membrane</td>
</tr>
<tr>
<td>Mechanism – endocytosis vs. carrier/transporter mediated</td>
</tr>
<tr>
<td>Cellular trafficking</td>
</tr>
<tr>
<td>Intracellular organelle distribution</td>
</tr>
<tr>
<td>Cytosol localization</td>
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<tr>
<td>Cellular metabolism</td>
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<tr>
<td>Fluorescence decay over time</td>
</tr>
<tr>
<td>Cell toxicity</td>
</tr>
<tr>
<td>Cell injury in necrosis, apoptosis</td>
</tr>
<tr>
<td>Surface membrane/blebbing</td>
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<tr>
<td>Mitochondrial function</td>
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<tr>
<td>Glomerular filtration rate determination</td>
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IUSM Cellular & Integrative Physiology
Mouhamad Alloosh
Michael S Sturek
NIH O’Brien Center for Advanced Renal Microscopic Analysis

- Develop new optical methodologies for renal investigators.

- Assist investigators in implementing these new techniques in their laboratories, or in the facilities of the Center.

Investigators interested in using the facilities of the Center can find details and application information at:

www.nephrology.iupui.edu/obrien