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### Establishing a Mouse Model for the Study of Podocyte Regeneration after Injury

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# **SENIOR THESIS APPROVAL**

This Honors thesis entitled

**“Establishing a Mouse Model for the Study of Podocyte  
Regeneration after Injury”**

Written by

**John A. Gomez**

and submitted in partial fulfillment of  
the requirements for completion of  
the Carl Goodson Honors Program  
meets the criteria for acceptance  
and has been approved by the undersigned readers.

Joe Jeffers, thesis director

Marty Perry, second reader

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Dr. Barbara Pemberton, Honors Program director

May 2, 2012

# Establishing a Mouse Model for the Study of Podocyte Regeneration after Injury

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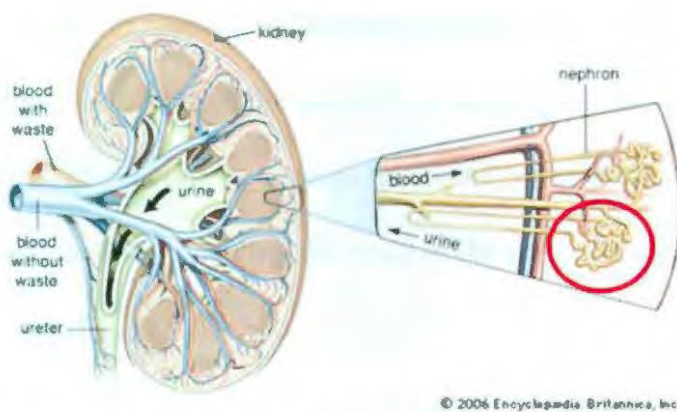
## Abstract

Podocytes are vital, specialized kidney cells that are post-mitotic and cannot proliferate.<sup>1</sup> Nevertheless, regeneration of podocytes after low-level ablation from a presently unidentified stem cell pool has been observed.<sup>2</sup> The objective of this study was to establish a mouse model for the study of this regeneration process. Two transgenic mice were developed, the first of which possessed a tomato-reporter transgene for the assessment of podocyte turnover. Adriamycin was used to induce podocyte damage in this mouse. The second transgenic mouse possessed the tomato-reporter transgene as well as a transgene allowing for the diphtheria toxin inducible (iDTR) ablation of podocytes.<sup>3</sup> Dosage tests were conducted to evaluate the effectiveness of these models in ablating podocytes. Both the Adriamycin and iDTR mouse models successfully induced podocyte damage, with the iDTR system being ~1500x more effective. The models exhibited podocyte damage far exceeding the threshold at which regeneration becomes impossible. Lower-level ablation is needed, and further iDTR dosage tests will be conducted to determine the optimal drug dosages.

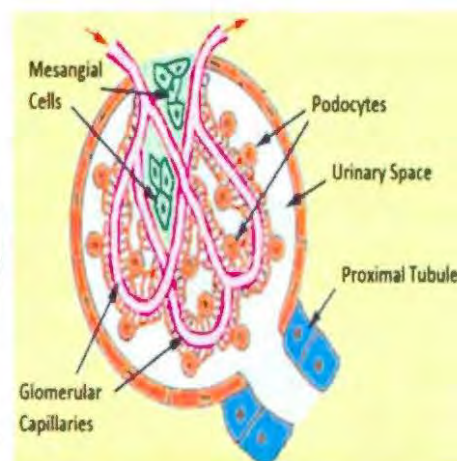
## Introduction

The primary purpose of the kidney is the filtration of the blood. This process allows for the removal of toxins and wastes and regulation of ions and blood volume, while also keeping vital blood proteins from being passed into the urine. Within the kidneys, blood filtration and

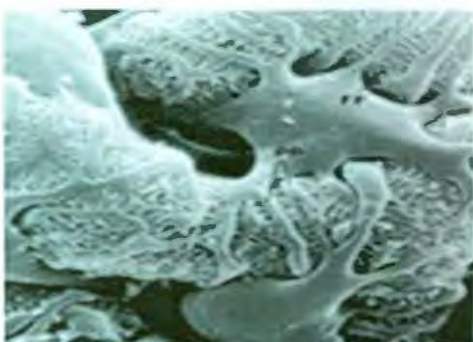
urine production occur in functional units called nephrons. The filtration step occurs within a structure called the glomerulus (See Figure 1) and, over the course of the lifetime of a healthy individual, 5 million liters of virtually protein-free urine are produced, meaning that 200,000 kg of protein are kept by the glomeruli in the blood.<sup>4</sup> The glomerular filter is tripartite, consisting of a fenestrated endothelium, glomerular basement membrane and, most importantly, highly specialized epithelial kidney cells called podocytes.<sup>4</sup> Podocytes cover the glomerular capillaries with interdigitating foot processes that form 40-nm-wide filtration slits, called slit-diaphragms<sup>4</sup> (See Figures 2-4), forming a sieve-like structure through which small molecules can pass, but proteins cannot.



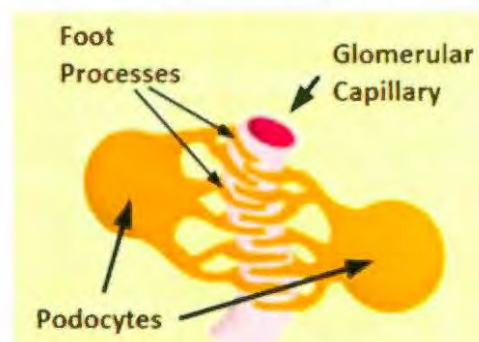
**Figure 1** The kidney, with glomerulus circled in red.



**Figure 2** The Glomerulus, showing the podocytes enveloping the glomerular capillaries which is collected by the tubules. Modified from <http://www.siumed.edu/~dking2/crr/images/glom1.jpg>



**Figure 3** Electron micrograph of podocytes and the foot processes.  
<http://www2.niddk.nih.gov/NR/rdonlyres/34D13CB4-1659-4F2A-AB59-E33C6E9192E2/0/podocytes.jpg>



**Figure 4** Podocyte with foot processes interdigitating across the surface of a glomerular capillary.  
 Modified from <http://www.siumed.edu/~dking2/crr/images/glom1.jpg>



Kidney disease is a significant health problem, currently constituting about 7% of the United States Medicare budget, a number that is expected to increase 50% by the year 2020.<sup>1</sup> Most renal pathologies that result in fatal kidney failure, or End Stage Renal Disease (ESRD), originate in the glomerulus as the result of severe podocyte loss and/or damage.<sup>1</sup> Podocytes are terminally differentiated, i.e. they cannot proliferate. Therefore, it has been concluded from normal podocyte population maintenance and the observed restoration of kidney function after minor injury, that low-levels of podocyte loss can be replenished via regeneration. This regeneration mechanism is of great interest to the nephrology community and much research is being conducted to understand the differentiation of podocytes from their presently unidentified progenitor stem cell pool.<sup>2</sup> However, when podocyte damage exceeds a certain threshold, ~30%, glomerulosclerosis ensues and podocyte regeneration becomes impossible. The objective of this study is to explore the viability of using Adriamycin or an iDTR system as a means of inducing low-level podocyte damage below the scarring threshold, allowing for podocyte regeneration, and, thereby, enabling the study of the podocyte regeneration mechanism.

## **Methods**

The podocyte injury model contains two primary components: 1) proteinuria, for the analysis of podocyte function, and 2) the tomato-reporter transgene to assess podocyte turnover (work has been done to quantify podocyte turnover using flow-cytometry, but these results are not shown here). As mentioned above, this experiment focused on Adriamycin and diphtheria toxin-induced podocyte damage.

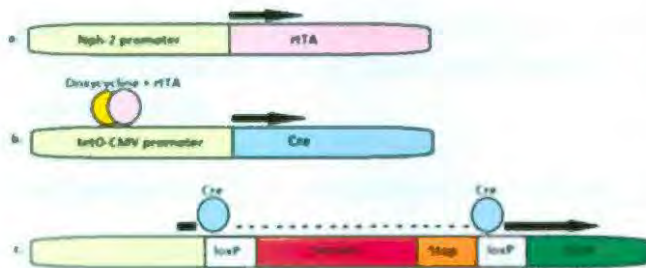
### **Proteinuria**

Proteinuria is a measurement of the amount of protein that escapes into the urine, and is the hallmark of podocyte damage. A proteinuria assay was conducted at regular intervals to

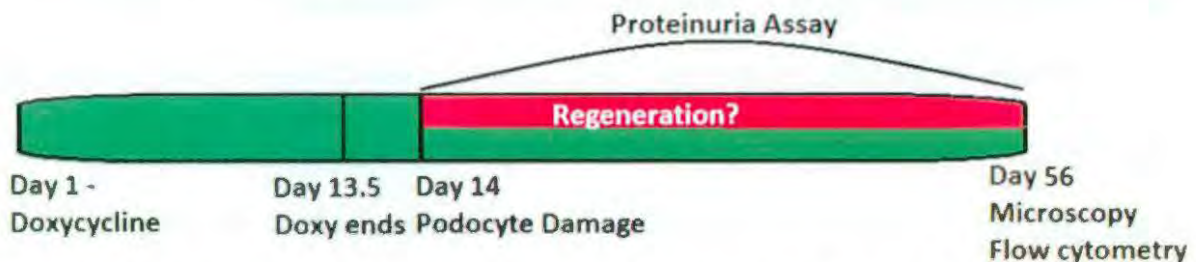
measure the ratio of albumin to creatinine in the urine. Plotting proteinuria as a function of time shows the change in kidney function, and allows the amount of podocyte loss and regeneration to be determined.

### Tomato-Reporter Transgene

All mice possessed the tomato reporter transgene described in Figure 5. The function of this transgene is that podocytes that develop while the mice are being treated with doxycycline express tomato, a red fluorescent protein. Podocytes that regenerate after doxycycline treatment ends express enhanced green fluorescent protein (EGFP). Fluorescence microscopy and flow cytometry (results not shown) were used to assess functionality of the transgene, and histological stains were conducted to examine kidney condition after testing. The general experimental scheme is shown in Figure 6.



**Figure 5:** A) The podocyte-specific Nph-2 promoter allows rtTA to be expressed constitutively in podocytes. B) Mice were fed doxycycline via their drinking water. The doxycycline/rtTA complex activates the tetO-CMV promoter, allowing Cre to be expressed in podocytes. C) Cre excises the tomato (red fluorescent protein) gene and stop codon at the loxP sites, resulting in the expression of EGFP.



**Figure 6** Mice were treated with doxycycline from conception (by treating the mother) through day 13.5 post-birth. Doxycycline cessation coincided with kidney maturation. Because podocytes do not continue to proliferate, all podocytes after this point are green until damage. At day 14, kidney damage is induced. From this point until the end of the study, proteinuria is monitored regularly. Any new podocytes that regenerate should express the red fluorescent protein, which was then assessed using the methods described.



### Adriamycin-Induced Podocyte Ablation

For the Adriamycin dosage test, mice were treated with 0  $\mu\text{g}$  Adriamycin/g bodyweight (3 mice), 10  $\mu\text{g}$  Adriamycin/g bodyweight (3 mice) and 15  $\mu\text{g}$  Adriamycin/g bodyweight (3 mice). Proteinuria was tracked throughout the course of the study by measuring the ratio of albumin to creatinine in urine samples weekly for 8 weeks.

### Diphtheria Toxin-Induced Podocyte Ablation

The iDTR mice possessed a transgene in addition to the tomato-reporter gene described above in Figure 5. This additional component is described below in Figure 7.

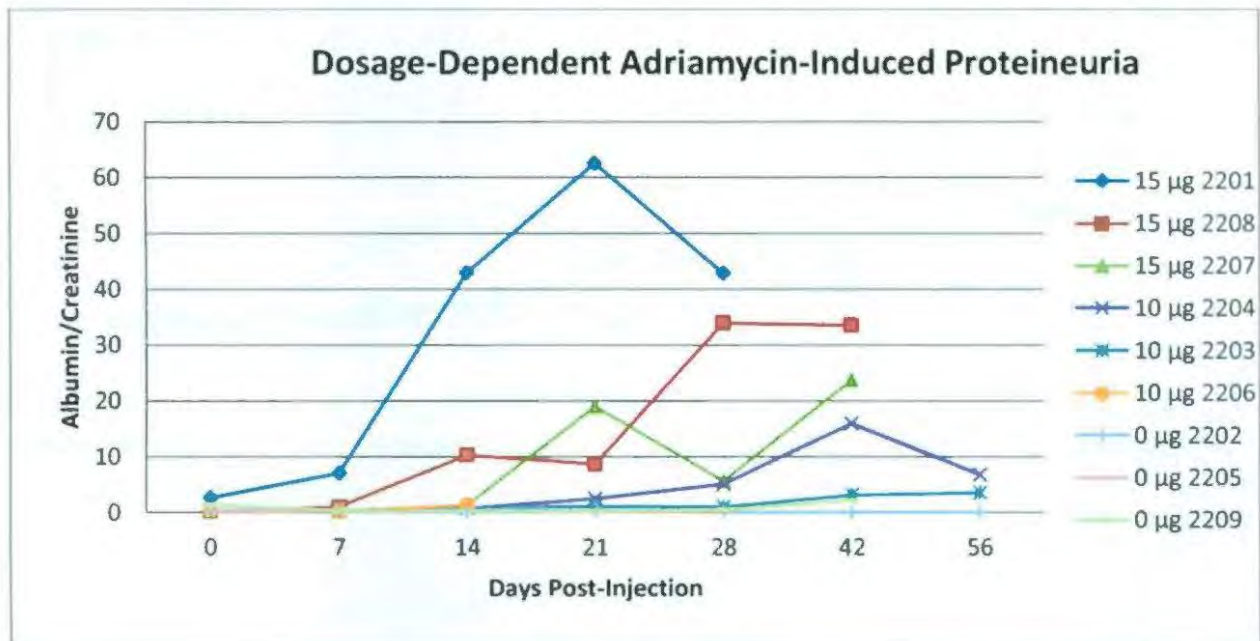


**Figure 7** The iDTR transgene drives the podocyte-specific expression of the iDTR receptor when the mice are treated with doxycycline (see Figure 5). Treatment with diphtheria toxin (DT) results in podocyte death.

The general experimental scheme here was the same as that shown in Figure 6. Intraperitoneal injection of DT and cessation of doxycycline were done after the completion of podocyte development in the kidneys. Mice were treated with 100 ng/g bodyweight DT (2 mice), 50 ng DT/g bodyweight (2 mice), 25 ng DT/bodyweight (1 mouse), 1 ng DT/g bodyweight (2 mice) and 0 ng DT/g bodyweight (3 control mice). Urine was collected from the mice at days 0, 1, 2, 3, 5, 10 and at weeks 2, 3, 4, 6 and 8. Fluorescence microscopy and Periodic acid-Schiff (PAS) stains were conducted with kidney sections to compare and assess the levels of kidney damage.

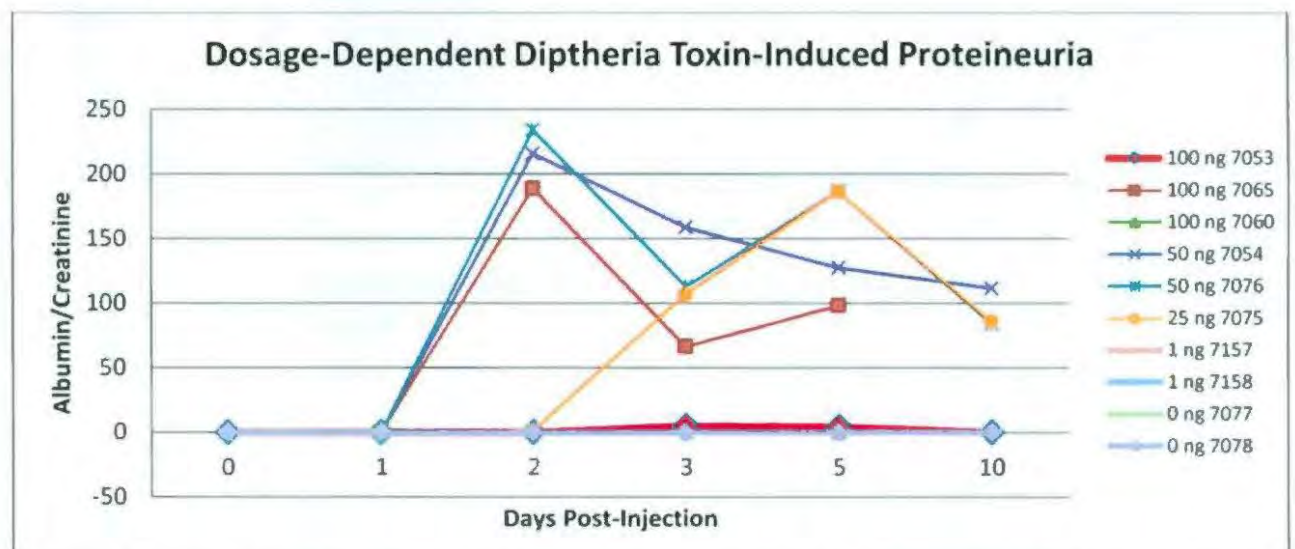
## Results

### Adriamycin-Induced Proteinuria



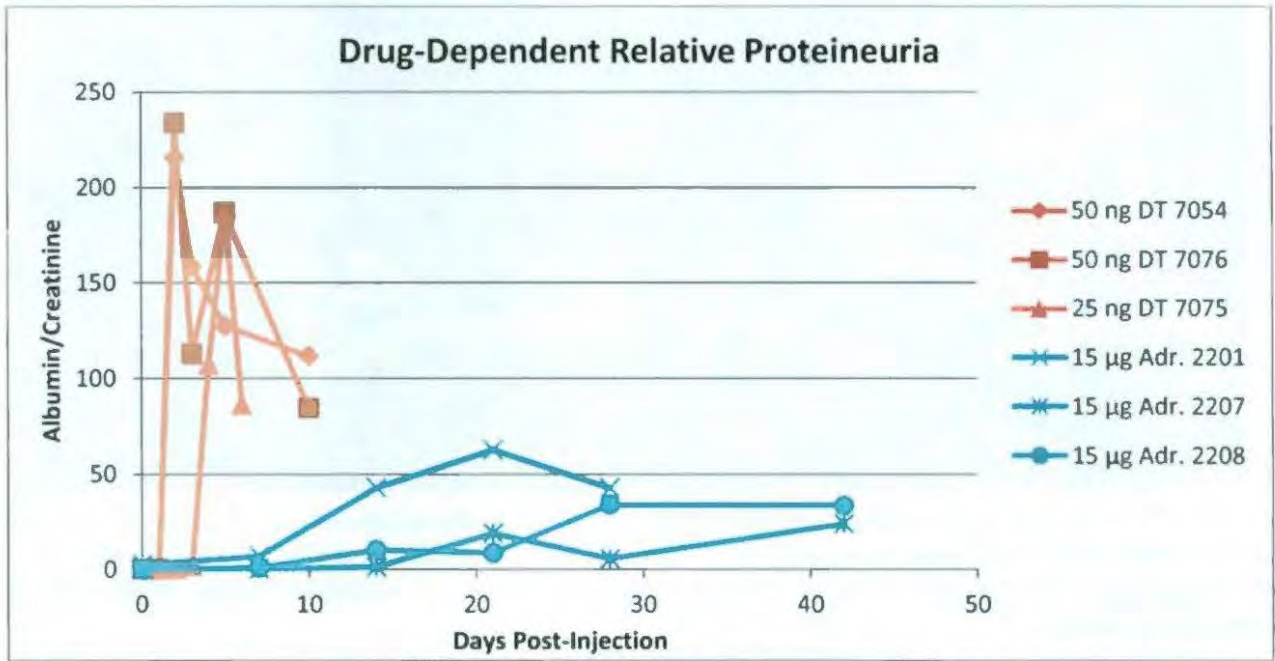
**Figure 7** Proteinuria (Albumin/Creatinine) vs. days post-Adriamycin injection for up to 56 days. The numerals in the legend (2201 etc.), are the mice identifiers. Several of the mice became very sick due to extreme proteinuria and were sacrificed before the end of the study.

### Diphtheria Toxin-Inducible Podocyte Ablation

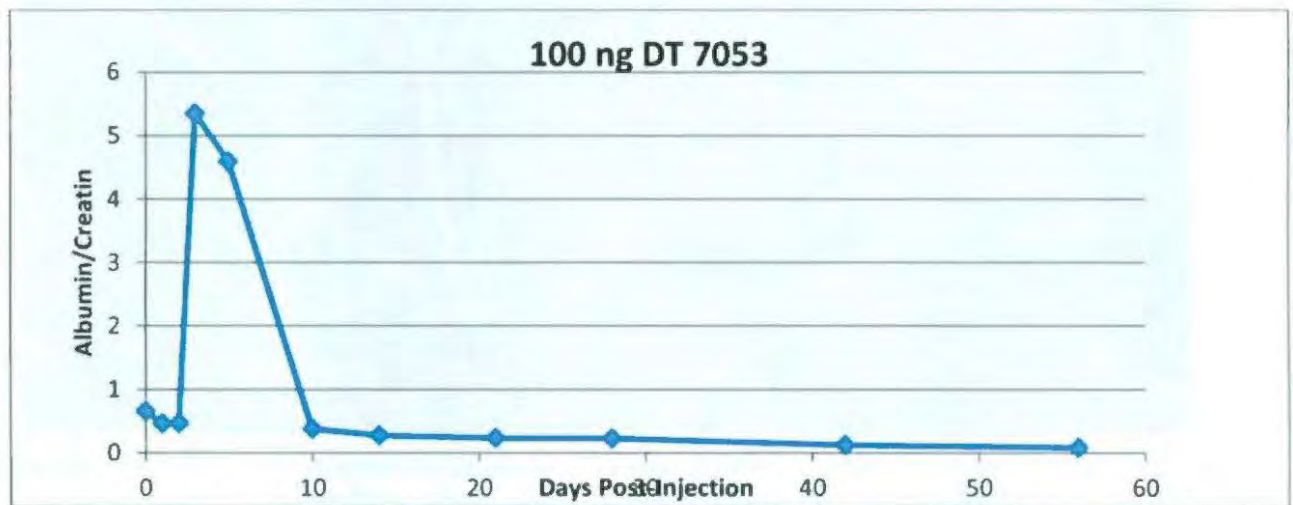


**Figure 8** Proteinuria (Albumin/Creatinin) vs. days post-DT injection for up to ten days. Most mice exhibited extremely high levels of proteinuria, became very sick and were sacrificed by day 10. The control mice were also sacrificed at this point so that the state of their kidney tissue could be compared with that of the test mice. One mouse, 100 ng 7053 (the red curve visible at the bottom of the graph), exhibited a low level of proteinuria that decreased over time (see Figure 5) and remained in good health throughout the course of the eight-week study.

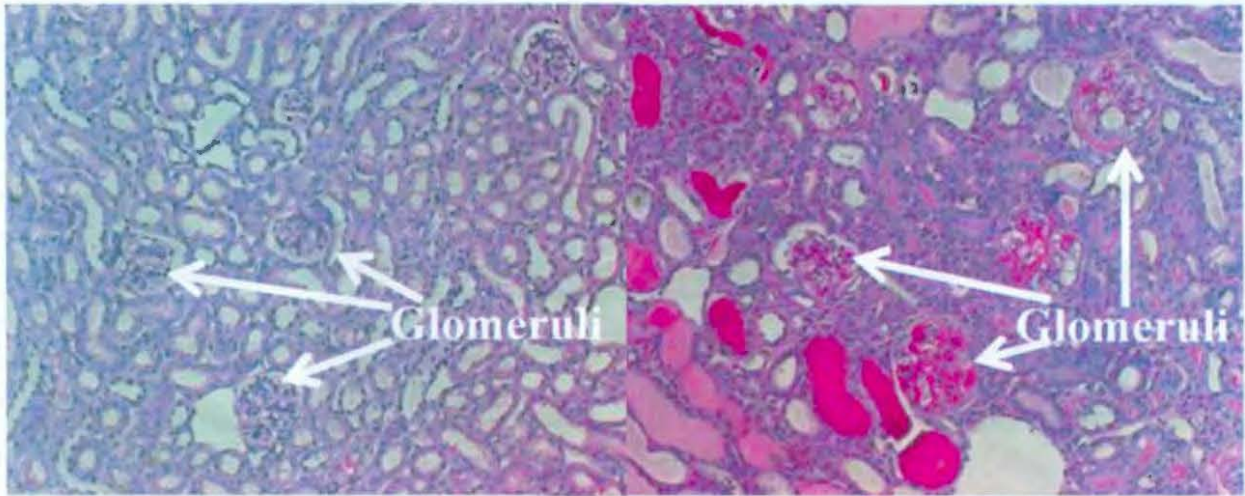




**Figure 9** Graph comparing the relative amounts of proteinuria (albumin/creatinine) induced by iDTR (red) and Adriamycin (blue).

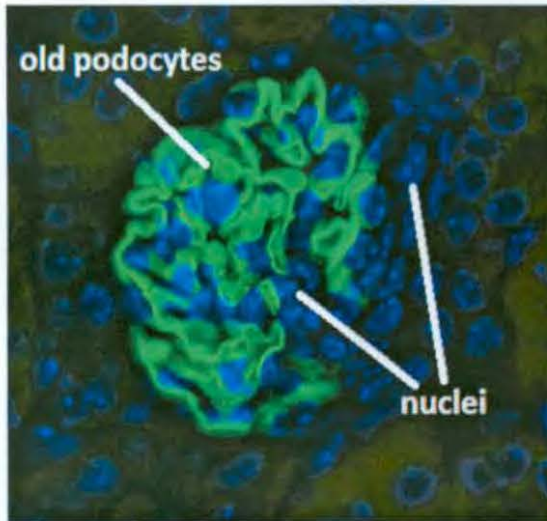


**Figure 10** Proteinuria (albumin/creatinine) vs. days post-injection for mouse 7053, treated with 100 ng diphtheria toxin/g bodyweight.

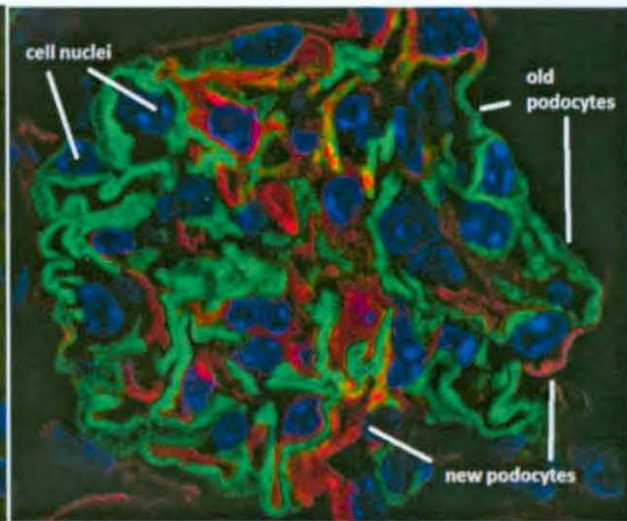


**Figure 11** 20x light microscopy image of PAS stain of DT Control Mouse 7060. Kidney tissue appears healthy.

**Figure 12** 20x light microscopy image of PAS stain of 100 ng DT/ g bodyweight mouse 7065, which exhibited massive proteinuria. High PAS positive stain (dark pink) corresponds with glomerulosclerosis and deposition of saccharide-rich immune-proteins.



**Figure 13** Fluorescence microscopy image of glomerulus from DT mouse 7060, which did not exhibit proteinuria. Green cells are “old” podocyte expressing EGFP. Cell nuclei, blue, stained with Hoechst.



**Figure 14** Fluorescence microscopy image of glomerulus from DT mouse 7053, which showed a decrease in proteinuria over time. Green cells are “old” podocyte expressing EGFP. New podocyte growth expresses the tomato reporter protein and is red. Cell nuclei, blue, stained with Hoechst.



## Discussion

It can be concluded from the albumin/creatinine measurements taken throughout the course of this study that both the iDTR and Adriamycin mouse models result in massive proteinuria (normal levels are below 2, see Figures 7 and 8). Many of the mice were sacrificed due to the illness result from the proteinuria. Although some of the proteinuria curves decline, this "decrease" in proteinuria should not be seen as a regain of kidney function. Rather, the scar tissue that develops around the glomerular capillaries was actually a more effective filter than the obliterated podocytes. High levels of scarring were confirmed by Figures 11 and 12, and it may be concluded that podocyte damage was induced well above the level at which regeneration was able to occur. For this reason, a second round of dosage was completed and the fine-tuning of the model is underway. However, as a much smaller amount of DT than Adriamycin is required (ng vs.  $\mu\text{g}$ ) to induce greater podocyte ablation, the iDTR mouse model will be the only one to be further tested. Most of the mice in the iDTR dosage test became very ill and had to be sacrificed by day 10 of the study. The control mice were also sacrificed at this point to allow for the comparison of tissue damage between these and the treated mice. Mouse 7053, which was the only mouse to regain health and kidney function and exhibit podocyte regeneration (see Figures 10 and 14), was one of two mice treated with the highest dosage of diphtheria toxin (100 ng/ g body weight). That this mouse exhibited much lower podocyte damage than the other mice is puzzling. After further testing, it should be clear whether an alternative drug delivery system is needed to obtain repeatable results.



## Acknowledgments

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