

## Progress report for the Cystinosis Research Foundation

**Title:** Treatment of cystinosis nephropathy using genetically modified adult stem cells in the murine cystinosis model

**PI:** Stephanie Cherqui and Daniel R. Salomon  
The Scripps Research Institute

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### I- Mice

#### 1- *Ctns*<sup>-/-</sup> mice

We have now a large colony of C57BL/6 *Ctns*<sup>-/-</sup> mice and we have achieved the necessary animal numbers to support the studies. We verified the integrity of *Ctns*<sup>-/-</sup> mice by measuring the cystine content in several organs. The *Ctns*<sup>-/-</sup> mice accumulate cystine in muscle, spleen, heart, liver and kidney (Table 1). The cystine measurements were performed by Jon Gangoiti at UCSD using a mass spectrophotometer (purchased with the support of the CRF). This experiment allowed Jon and I to optimize the parameters for cystine measurements in murine tissues.

	nmol/mg prot. ½ cystine	ratio accumulation KO/wt
wt muscle	0.002	51
wt spleen	0.018	67
wt heart	0.032	25
wt liver	0.001	1642
wt kidney	0.040	43
KO muscle	0.108	
KO spleen	1.207	
KO heart	0.794	
KO liver	2.444	
KO kidney	1.723	

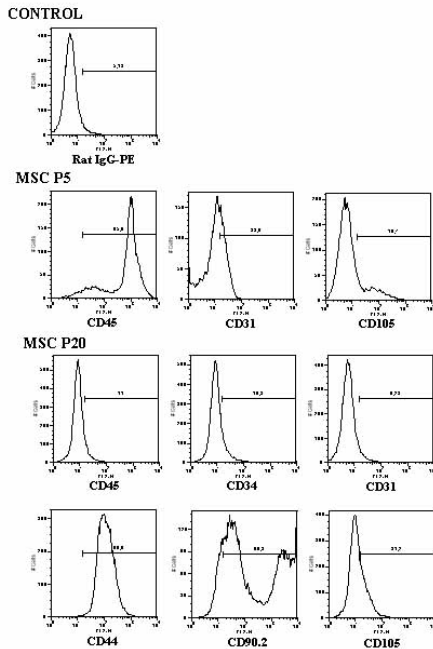
**Table 1:** Cystine measurement in the muscle, spleen heart, liver and kidney of a wildtype and *Ctns* Ko mice.

### II - Transplantation

We performed several bone marrow stem cell (BMSC) transplantations in *Ctns*<sup>-/-</sup> mice. BMSC were isolated from our colony of mice transgenic for the Green Fluorescent Protein (GFP) expressed under the control of a ubiquitous  $\beta$ -actin promoter (C57BL/6-Tg(ACTB-EGFP)10sb/J, Jackson Laboratory). The premise is that after transplantation, any cell derived from these BMSC will be fluorescent and detectable using flow cytometry or microscopy. For controls, we isolated BMSC from *Ctns*<sup>-/-</sup> mice. The premise is that we need to exclude the possibility that simply transplanting BMSC might have some unexpected impact on the *Ctns*<sup>-/-</sup> mice. We transplanted either BMSC or mesenchymal stem cells (MSC). We performed the cell injections by two methods: tail vein injection in sublethally irradiated mice or ureteral injections into the ureter of the right kidney (in which case the left kidney serves as the internal control).

#### 1- Cells

Two kinds of stem cells were transplanted to test which one is the more efficient to generate renal cells. First, the BMSC represent the heterogeneous population of cells contained in the whole bone marrow. These were freshly harvested from the mice and



**Figure 1:** Flow cytometry analysis of the mesenchymal stem cells (MSC) at passage 5 (P5) and passage 20 (P20). At P5, the cells were expressing HSC markers as CD45 and CD31. At P20, the MSC express only MSC-specific markers (CD44, CD90.2, CD105) and not the HSC markers (CD45, CD34, CD31).

injected into *Ctns*<sup>-/-</sup> mice. Two independent experiments have been performed with BMSC, n=3 animals/condition x 2 experiments for a total of 6 animals per condition as follows:

- *Ctns*<sup>-/-</sup> mice receiving GFP<sup>+</sup> BMSC via tail vein injection
- *Ctns*<sup>-/-</sup> mice receiving GFP<sup>+</sup> BMSC via ureteral injection
- *Ctns*<sup>-/-</sup> mice receiving *Ctns*<sup>-/-</sup> BMSC via tail vein injection
- *Ctns*<sup>-/-</sup> mice receiving *Ctns*<sup>-/-</sup> BMSC via ureteral injection

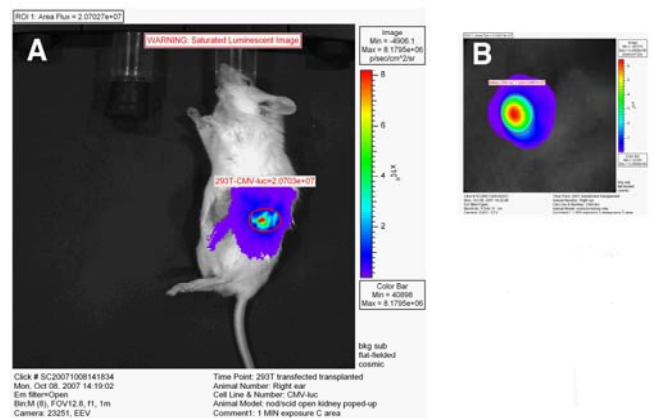
The second kind of stem cells used for transplantation were the MSC. Indeed, some authors have shown that these cells are more efficient to generate renal cells as discussed in the original proposal. We obtained MSC by culturing BMSC from GFP transgenic mice for 2 months, changing the media every 3 days to remove non-adherent cells. As clearly shown in Figure 1, after 12 passages the stem cells stopped expressing hematopoietic stem cell (HSC) markers (CD45, CD31, CD34) and started expressing MSC markers (CD90.2, CD44, CD105). Two independent experiments have also been performed using these cells. For one of these experiments, the MSC have been genetically

modified to express a Luciferase gene. Luciferase allows us to follow the cells in live mice using the IVIS Imaging System 200 Series (Xenogen). Each experiment included:

- 3 *Ctns*<sup>-/-</sup> mice receiving GFP<sup>+</sup> MSC ( $\pm$  Luciferase) via tail vein injection
- 3 *Ctns*<sup>-/-</sup> mice receiving GFP<sup>+</sup> MSC ( $\pm$  Luciferase) via ureteral injection

## 2- Injection

The cells have been injected into the mice using two different techniques. The first technique is injection via the tail veins of the mice after lethal (for BMSC) or sublethal (for MSC) irradiation. Thus, the transplanted cells replace all or part of the host hematopoietic or mesenchymal stem cells. The premise is that these stem cells will migrate to the kidney if there is ongoing renal cell injury and death. The second technique, ureteral injection, is aimed to target more precisely and more



**Figure 2:** Proof of concept for cell delivery to the kidney via ureteral injection. (A) NOD/SCID mouse after ureteral injection of 293T-Luciferase. The Fluorescence can be observed in the kidney area using the IVIS Imaging system. (B) Kidney after extraction, proving that the cells are in the kidney.

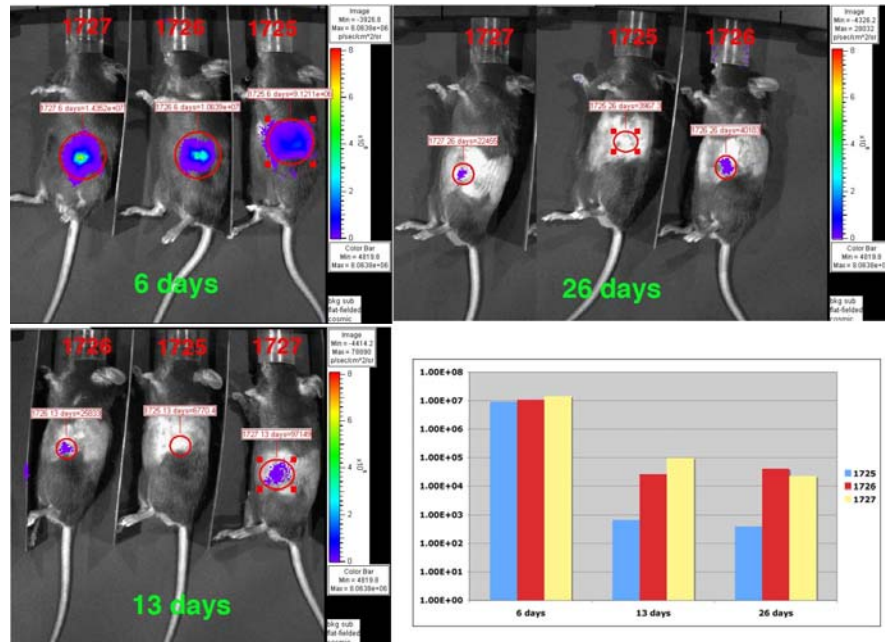
efficiently the kidney. Moreover, this procedure would be the best to apply to cystinosis patients; it would be less invasive and less dangerous for the patient than a renal arterial injection. This technique has been shown to deliver virus vectors more efficiently to the kidney than intra-parenchymal or intra-arterial injections [Gusella et al., 2002a; Gusella et al., 2002b; Lai et al., 1997]. However, this method has never been used to inject cells. Therefore, as a proof of concept, we injected 293T (human embryonic renal cell line) expressing luciferase via ureteral injection in the right ureter of NOD/SCID mice. Four days later, we could observe Luciferase fluorescence in the area of the kidney in live mice (Figure 2A). To prove that the fluorescence came from the kidney, we sacrificed the mice and extracted the right kidney, which was clearly positive for Luciferase (Figure 2B).

### 3- Results

The mice transplanted were between 8 to 12 weeks old for each experiment. At 8 weeks post-transplantation, we measured urea and creatinine in the serum and urine as well as alkaline phosphatase in serum. As expected, no major difference was observed between the mice transplanted with GFP<sup>+</sup> BMSC and *Ctns*<sup>-/-</sup> BMSC as no renal injury is observed at that age in *Ctns*<sup>-/-</sup> mice. However, it is important to note that the procedure was well tolerated by the animals and there were no problems encountered post transplantation if the mice successfully engrafted after irradiation. Therefore, we are leaving the mice to get older and we will measure their renal function at different time points with the assumption based on previous studies that renal injury in these animals will begin to accumulate after 6 months. We will eventually sacrifice the mice to verify

and quantify the presence of GFP<sup>+</sup> renal cells in the kidneys of *Ctns*<sup>-/-</sup> mice transplanted with either GFP<sup>+</sup> BMSC or GFP<sup>+</sup> MSC. We will also compare the cystine content and the renal injury of the kidneys of *Ctns*<sup>-/-</sup> mice receiving the GFP<sup>+</sup> BMSC or GFP<sup>+</sup> MSC to the results in the mice transplanted with *Ctns*<sup>-/-</sup> BMSC.

As shown in Figure 3, mice transplanted via a



**Figure 3:** Following of mesenchymal stem cells expressing Luciferase after ureteral injection in *Ctns*<sup>-/-</sup> mice. The cells can be observed in live animals using the IVIS imaging system at 6, 13 and 26 days post injection. The histogram shows the total flux intensity of Luciferase in each mouse.

ureteral injection with MSC expressing luciferase demonstrated luciferase fluorescence in the kidney area 6 days after the injection (n=3). Luciferase fluorescence was observed in 2 of the 3 mice at 13 and 26 days post injection. The fluorescence intensity decreased between 6 and 13 days, which is not surprising because a substantial number of cells die after injection. However, between 13 and 26 days, the fluorescence intensity in the positive mice (# 1726 and 1727) remains stable (Figure 3). These results support our hypothesis the cells are integrated into the kidney. The mice are still alive and will be monitored every other week for at least two more months.

However, we strongly believe that we need a better model for renal injury so that there is a constant force driving potential stem cells to migrate to the kidney and repair an ongoing injury.

### III- Optimization of the cystinosis model for nephropathy

As described in the original project proposal, we hypothesize that the more progressive the kidney injury, the more robust the migration and engraftment of the bone marrow stem cells will be. Therefore, to test the efficiency of stem cell transplantation in cystinosis, it is important to enhance the nephropathy observed in the murine cystinosis model to obtain a proximal tubulopathy or frank renal failure as observed in human cystinosis patients.

#### 1- Generation of a drug-inducible proximal tubulopathy and renal failure via a novel transgenic mouse

We generated the genetic constructs for engineering a drug-inducible proximal tubulopathy and renal failure in mice. We subcloned a highly active form of the viral thymidine kinase gene (vTK) into a plasmid under the control of a proximal tubular cell specific promoter (Figure 4). The rationale is that treatment with the drug, ganciclovir, will kill the cells where vTK is expressed. To follow the expression of the construct, a marker gene, DsRed, is subcloned downstream of vTK and the two genes are linked by an IRES (Internal Ribosomal Entry Site) sequence. Thus, the same promoter will drive the expression of both vTK and DsRed. We added a Nuclear Localization Site (NLS) at the end of the DsRed gene to direct the expression of DsRed primarily to the nucleus. Therefore, the intensity of the fluorescence of DsRed will be amplified and easily recognized in histology sections.

Based on a literature search, we chose two candidates for proximal tubular cell specific promoters: the promoter of the 25-hydroxyvitamin D3-1 $\alpha$ -hydroxylase gene,



**Figure 4:** Genetic construct generated for drug-inducible proximal tubulopathy and renal failure. The constructs contain DsRed reporter gene with a Nuclear Localization Site (NLS) in 3', preceded by an Internal Ribosomal Entry Site (IRES) and a viral Thymidine kinase gene (vTK). The expression of these genes are under the control of either a kidney or proximal tubules specific promoter.

CYP27B1, and the promoter of the glucose transporter, Sglt2. We generated the corresponding constructs and tested them *in vitro* by transfecting three different cell lines: NIH 3T3 (murine fibroblasts), MDCK (canine renal distal tubular cells) and LLC-PK1 (pig renal proximal tubular cells). Our goal was to verify if either one of the constructs, pIS-CYP or pIS-Sglt2, has a promoter specific enough to get DsRed expression only in the nuclei of the LLC-PK1 cells and to provoke cell death of LLC-PK1 in presence of the drug, ganciclovir. We will then use that construct to generate transgenic mice expressing this drug-inducible toxin specifically in the proximal tubule cells of the kidney. Thus, treatment of these mice with the drug, ganciclovir, should produce a proximal tubulopathy and eventually renal failure. If successful, these vTK transgenic mice will then be bred with the *Ctns*<sup>-/-</sup> mice to generate a controllable proximal tubulopathy in the murine cystinosis model. The current data suggests that the Sglt2 promoter is optimal for our purposes and we are finishing the final experiments.

We also decided to generate a construct that would lead to a general renal failure by using a kidney specific promoter that drives expression in different kinds of renal cells. We chose the promoter of CDH16 (ksp-cadherin), which is expressed in tubular epithelial cells in the kidney (distal and proximal), collecting ducts and thick ascending limbs of Henle's loop. Based on the literature, we tested two different sizes of the CDH16 promoter, one at 300 bp and one at 1300 bp. The larger one showed more specificity for renal cells. Our testing of this construct *in vitro* has been very promising and we are now doing final validations.

## **2- Decreasing resistance to oxidative stress with SOD deficiency**

Several lines of evidence suggest that cystine accumulation in the lysosomes of renal tubules results in increased oxidative stress, which results in cell dysfunction and tissue injury. The lack of tubulopathy in the *Ctns*<sup>-/-</sup> mice might be due to a better resistance of these animals to oxidative stress than human patients. Our hypothesis is that we can significantly enhance the progression of renal injury in our cystinosis model by decreasing the resistance of these animals to oxidative stress. Oxidative stress and mitochondrial dysfunction results in decreased of ATP levels and increased of superoxide dismutase (SOD) production, an antioxidant protein protecting the cells against oxidative stress. Therefore, to test our hypothesis we will inhibit SOD production in *Ctns*<sup>-/-</sup> mice by backcrossing them with SOD-deficient mice.

This work is done in collaboration with Dr Jeffrey Friedman (The Scripps Research Institute, La Jolla) who has the SOD1- and SOD2-deficient mice. SOD1 is a cytosolic form and SOD2 is a mitochondrial form of SOD. The backcrosses of the *Ctns*<sup>-/-</sup> mice with *SOD1*<sup>-/-</sup> mice and *SOD2*<sup>+/-</sup> mice (*SOD2*<sup>-/-</sup> are lethal) are currently going on. We want to obtain *Ctns*<sup>-/-</sup> *SOD1*<sup>-/-</sup> and *Ctns*<sup>-/-</sup> *SOD2*<sup>+/-</sup> mice, which will then be tested for their renal phenotype. These mice are difficult to obtain because the SOD knock-out are smaller and less efficient for the reproduction.

We obtained our 2 first double knock-out *Ctns*<sup>-/-</sup> *SOD1*<sup>-/-</sup> mice 2 months ago. We tested their renal function by measuring creatinine and urea levels in the urine and serum of the mice and alkaline phosphatase in the serum. Two *SOD1*<sup>-/-</sup> and two *Ctns*<sup>-/-</sup> mice were used as controls. Urea and creatinine of the *Ctns*<sup>-/-</sup> *SOD1*<sup>-/-</sup> mice were normal and

comparable to the ones obtained for *SOD1<sup>-/-</sup>* and *Ctns<sup>-/-</sup>* mice (Table 2). However, the alkaline phosphatase levels were increased in *Ctns<sup>-/-</sup> SOD1<sup>-/-</sup>* mice compared to *SOD1<sup>-/-</sup>* and *Ctns<sup>-/-</sup>* mice (Table 2). These data are encouraging because the increased alkaline phosphatase level in serum is one of the markers for proximal tubulopathy. Serum urea and creatinine levels are increased only when the kidney injury reaches the end-stages of renal failure. We are currently measuring the amino-acid levels in the urine of these mice. The premise is that if the *Ctns<sup>-/-</sup> SOD1<sup>-/-</sup>* mice really are presenting a proximal tubulopathy, we should observe an increase of amino acids in their urines. We will also measure the phosphate, calcium, sodium, potassium and chloride levels in the urine and serum of these mice, other indicators for proximal tubulopathy.

However, these results are only preliminary. They represent only two mice for the moment. We expect to have enough double knock-out *Ctns<sup>-/-</sup> SOD1<sup>-/-</sup>* mice for our studies in the next one or two months.

Mice	Date of Birth	Sex	Genotype	Creatinine in serum (mg/dL)	Creatinine in urine (mg/dL)	Creatinine clearance (ml/min)	Urea in serum (mmol/L)	Urea in Urine (mmol/L)	Alkaline Phosphatase in serum (IU/L)
1731	10/8/07	M	Ctns <sup>-/-</sup> control	0.53	57.84	0.092	8.81	72.87	83.82
1732	10/8/07	M	Ctns <sup>-/-</sup> control	0.61	59.41	0.035	9.82	75.70	64.85
2401	10/16/07	M	Ctns <sup>-/-</sup> SOD1 <sup>-/-</sup>	0.53	53.22	0.032	10.93	72.45	156.15
2402	10/16/07	M	Ctns <sup>-/-</sup> SOD1 <sup>-/-</sup>	0.54	45.38	0.025	12.02	70.93	154.67
2476	8/1/07	M	SOD1 <sup>-/-</sup> control	0.46	51.92	0.025	11.72	70.08	51.97
2490	8/1/07	M	SOD1 <sup>-/-</sup> control	0.49	ND	0.000	10.24	ND	109.56

**Table 2:** Serum and plasma analyses for renal injury of the 2 double knock out *Ctns<sup>-/-</sup> SOD1<sup>-/-</sup>* mice compared to *SOD1<sup>-/-</sup>* and *Ctns<sup>-/-</sup>* mice.

### III- A new direction for the project: Gene therapy for cystinosis

We decided to test in parallel a new strategy to find a cure for cystinosis, focusing on the nephropathy as a proof of concept. In the original animal protocol we described a stem cell-based treatment for cystinosis. We now want to try a gene therapy-based treatment for this disease using the adeno-associated virus (AAV) as a gene delivery vector. These two methods are promising and will be tested in parallel to determine which one will be used in clinical application. AAV has the advantage of delivering the transgene (in our case CTNS) to a wide range of tissues with long-term expression and a lack of tissue pathogenicity. AAV has already been tested in preclinical studies in a large number of metabolic diseases and at least 20 clinical trials have been done or are presently underway in several hundred patients.

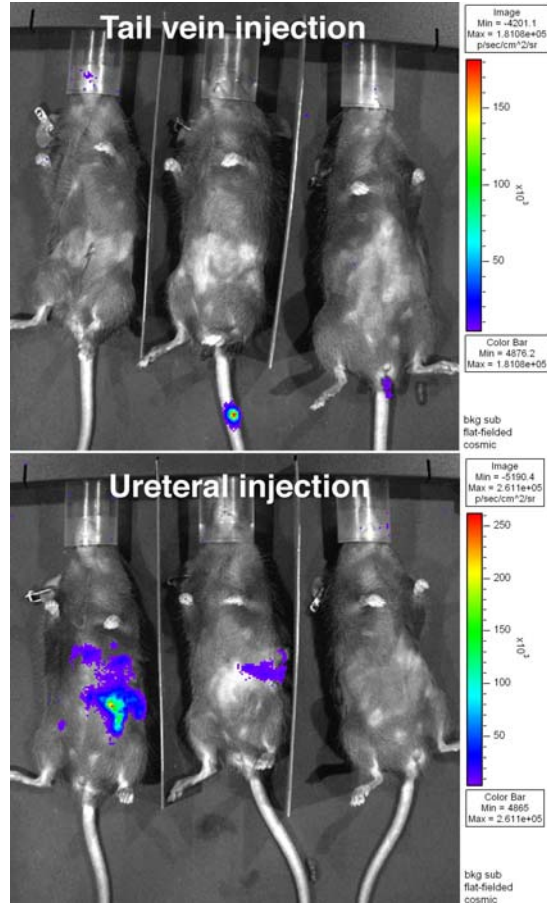
This project is being done in collaboration with Dr. Jude Samulski, an international expert on AAV vectors, and Director of the Gene Therapy Center (University of North Carolina at Chapel Hill). This facility ensures that investigators can have gene vectors available in the quality and quantities needed for preclinical or clinical studies. Research in his laboratory has centered on adeno-associated virus (AAV) in order to exploit the unique

features of this virus to develop an efficient viral vector system for use in human gene therapy. Several clinical trials for a variety of genetic diseases such as Duchenne Muscular Dystrophy are currently being done using vectors produced by the Gene Therapy Center.

The most commonly used AAV vector in clinical trials is the AAV serotype 2 because it has a well established safety and efficacy and it does not generate significant immune reactions [Wu et al., 2006]. Moreover, AAV2 can successfully and efficiently transduce kidney cells [Koeberl et al., 2007; Takeda et al., 2004; Wu et al., 2006].

Our goal is to generate and test AAV serotype 2 expressing CTNS in our *Ctns*<sup>-/-</sup> mice. We want an AAV vector that can efficiently target the kidney but also other organs such as liver, muscle, pancreas and brain that are known to be involved in cystinosis. We will perform two types of injection of the vectors, a tail vein injection and a kidney injection via the ureter. These two procedures will mimic the potential options for a clinical procedure by comparing a systemic injection of the vector to target most of the organs vs. the injection in the ureter to specifically target the kidney. To determine the efficiency of AAV2-CTNS on the kidney and other organs, we will study the treated animals for the presence of CTNS gene in the different tissues and the impact of the treatment will be determined by histology, quantifying cellular anomalies and cystine crystals and by cystine measurements as described in the original proposal.

To develop such a project, we will have to submit another grant to The Cystinosis Research Foundation because each production of AAV2-CTNS virus by the Gene Therapy Center will be quite expensive as well as the analyses (qPCR, histology, cystine measurement). However, we will first develop the proof of concept and test the efficiency of the virus transduction in different targeted organs as well as determine the stability of the transgene in animals. To do so, we have already injected AAV2 vectors expressing the reporter gene Luciferase (AAV2-Luc) via the tail veins or ureters of *Ctns*<sup>-/-</sup> mice. By these approaches, we can observe the cells transduced in live animals using the IVIS imaging system and determine the stability of the transgene expression.



**Figure 5:** AAV2-Luciferase injection in *Ctns*<sup>-/-</sup> mice. The upper panel shows the mice that got the tail vein injection of the virus, the particles can be observed only at the site of the injection. The lower panel shows the mice that got the ureteral injection of the virus, the fluorescent particles can be observed in the kidney for 2 of the 3 mice.

Thus, we recently injected  $1 \times 10^{12}$  AAV virus particles per mouse in 3 *Ctns*<sup>-/-</sup> mice via ureteral injection and 3 *Ctns*<sup>-/-</sup> mice via tail vein injection. Four days after the injection via the tail veins, luciferase could not be observed in the body of live mice except at the point of injection in the tail (Figure 5). However, this is very early for AAV expression of protein and the virus particles are distributed widely in the animal's tissues. Over time, AAV protein production should increase and it is still too early to conclude anything. In contrast, luciferase fluorescence is readily detected in the area of the kidneys in the animals injected via the ureters in 2 of the 3 mice (Figure 5). These mice will be kept alive for at least 3 months and monitored every over week for the presence of fluorescence. They will then be sacrificed to precisely determine the quantity and the phenotype of the cells infected.

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