

**PROGRESS REPORT SUMMARY - Humanized Mouse Models of Diabetic Nephropathy****SPECIFIC AIMS (OF ORIGINAL TWO-YEAR APPLICATION)**

Small immunodeficient animal models of diabetic nephropathy are needed to assess the impact of human cell populations on diabetic kidney complications. Immunocompetent mice bearing a mutated leptin receptor gene develop obesity, insulin resistance, and chronic hyperglycemia as they age. However, they fail to develop robust nephropathy. It has been reported that introduction of a mutated nitric oxide synthase 3 (*Nos3*) gene leads to accelerated and robust nephropathy in immunocompetent diabetic mice. However, these immunocompetent mice will reject human cells and cannot be used to test the potential of human cell-based therapies to impact diabetic complications. We have recently developed immunodeficient NOD-*scid* *IL2r $\gamma$ <sup>null</sup>* (NSG) mice that readily engraft with human cells and tissues, including hematopoietic stem cells, mesenchymal stem cells, and embryonic stem cells - cell populations under consideration as treatment for diabetic complications. We propose to develop three new immunodeficient stocks of mice that represent type 2 diabetes, nephropathy, and diabetic nephropathy, and can be readily engrafted with human cells: 1) NSG mice bearing a mutation in the leptin receptor (*lepr<sup>db/db</sup>*, abbreviated *db/db*, type 2 diabetes model), and 2) NSG mice bearing a mutated *Nos3* gene (*Nos3<sup>null</sup>*, nephropathy model). We will then intercross these two NSG stocks of mice to 3) generate NSG-*Nos3<sup>null</sup> db/db* (diabetic nephropathy model) mice. We have recently generated NSG-*db/db* mice that are now available and propose to generate NSG-*Nos3<sup>null</sup>* mice using a speed congenic marker assisted backcrossing approach. At the end of the first year of this P&F, we will have 1) characterized the development of obesity, diabetes, and nephropathy (or lack thereof) in NSG-*db/db* mice, validating this new model of type 2 diabetes, 2) be at the N3 backcross generation of NSG-*Nos3<sup>null</sup>* mice. In year 2, we will finish the backcross, and intercross with NSG-*db/db* mice to generate the stock of most interest, the NSG-*Nos3<sup>null</sup> db/db* mouse strain. This mouse will be available to the AMDCC for screening for the development of diabetic nephropathy and for its ability to support human cell engraftment and cell-based therapy.

We originally proposed three aims that will provide important new animal models for the AMDCC.

**Aim 1: Validation of the stock of NSG-*db/db* mice**

These mice will be evaluated for the development of obesity, hyperglycemia, and nephropathy.

**Aim 2: Generation of NSG-*Nos3<sup>null</sup>* mice using a speed congenic approach**

We will generate NSG-*Nos3<sup>null</sup>* mice using a marker assisted speed congenic approach. These normoglycemic mice will be evaluated for development of nephropathy.

**Aim 3: Generation of NSG-*Nos3<sup>null</sup> db/db* mice by intercrossing NSG-*db/db* with NSG-*Nos3<sup>null</sup>* mice**

We will complete development of the backcrossing of the *Nos3<sup>null</sup>* allele onto the NSG strain and intercross NSG-*db/db* and NSG-*Nos3<sup>null</sup>* mice to generate double mutant diabetic NSG-*Nos3<sup>null</sup> db/db* mice that are expected to exhibit robust diabetic nephropathy and support engraftment with human cells.

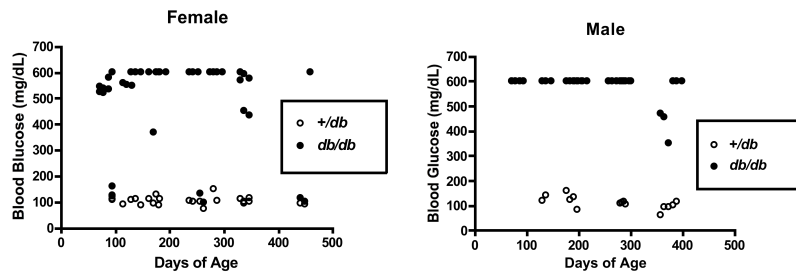
A Progress Report for Aims 1 and 2 followed by a description of studies to be carried out in Aim 3 is presented.

**Progress on Aim 1. Validation of the stock of NSG-*db/db* mice**

The NSG-*db/db* strain was generated in our laboratory by initially mating NOD-*Lep<sup>db5J</sup>/+* mice (JAX #004939) with NOD.Cg-*Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ* (JAX #005557, abbreviated NSG) mice and then intercrossing the NSG +/*db* sibs. Mice are genotyped for the *db<sup>5J</sup>* mutation using pyrosequencing to detect the G>T base change. The full protocol can be found at The Jackson Lab website:

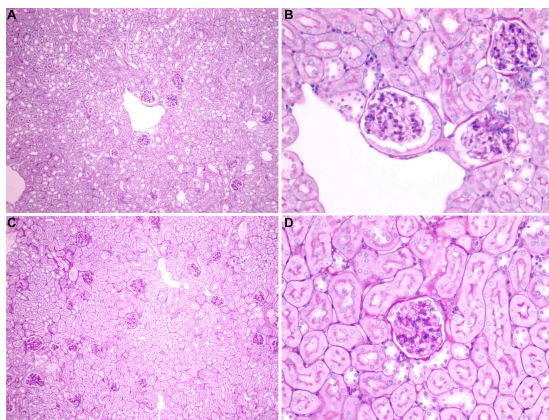
[http://jaxmice.jax.org/protocolsdb/f?p=116:2:2161950001919406::NO:2:P2\\_MASTER\\_PROTOCOL\\_ID,P2\\_JRS\\_CODE:2113,004939](http://jaxmice.jax.org/protocolsdb/f?p=116:2:2161950001919406::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:2113,004939) . NSG-*db/db* female and male mice develop hyperglycemia as they age

(**Figure 1**). These mice also show the obesity characteristic of *db/db* mice (25.8± 0.3gm for 8-11-week-old female NSG-*db/db* vs. 20.0± 0.3gm NSG +/*db* and +/+ littermate controls, p<0.01).



**Fig. 1** Blood glucose levels in NSG-*db/db* and littermate control (*+/db* and *+/+*) mice.

In order to determine whether NSG-*db/db* mice develop spontaneous renal changes, kidneys from 1 to 13 month-old NSG-*db/db* and littermate control mice were fixed in 10% neutral buffered formalin (NBF) and processed for routine staining (H&E and PAS). There were no marked abnormalities in the kidneys of young (data not shown) or aged NSG-*db/db* mice (**Figure 2**).



**Fig 2** Sections of kidneys from NSG-*db/db* and littermate control males at 13 months of age. A,B NSG-*db/db*. C,D NSG littermate control. A,C x 100. B,D x 400. PAS.

Kidney sections were scored blindly by a Board Certified veterinary pathologist. As shown in **Table 1**, there were no significant pathological changes in NSG-*db/db* kidneys.

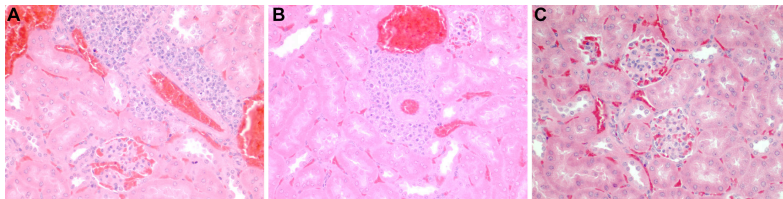
**Table 1.** Scoring of pathological changes in PAS-stained kidney sections from NSG-*db/db* and littermate control mice.

Genotype	Age (Days)	Sex	Glomeruli	Tubules	Interstitialium	Arterioles	Overall grade
<i>db/db</i>	404	M	1	0	0	0	0
<i>+/db</i>	404	M	1	0	0	0	0
<i>db/db</i>	380	M	1	0	0	0	0
<i>+/db</i>	380	M	0	0	0	0	0
<i>db/db</i>	353	F	1	0	0	0	0
<i>+/db</i>	353	F	1	0	0	0	0
<i>db/db</i>	353	F	1	0	0	0	0
<i>+/db</i>	353	F	1	1	0	0	1
<i>db/db</i>	306	M	0	0	0	0	0
<i>+/+</i>	306	M	0	0	0	0	0
<i>db/db</i>	303	M	0	0	0	0	0
<i>+/db</i>	303	M	0	0	0	0	0
<i>db/db</i>	264	F	1	0	0	0	0
<i>db/db</i>	264	F	0	0	0	0	0
<i>db/db</i>	64	F	0	0	0	0	0
<i>+/+</i>	64	F	0	0	0	0	0
<i>db/db</i>	64	M	1	0	0	0	0
<i>+/+</i>	70	M	1	0	0	0	0

The sections were graded as follows: Four major structures of the kidney were graded on a 0-4 scale. 0 represents normal and 4 severe changes. At least 10 glomeruli were examined for each animal. The overall grade represents all the changes and takes into account the proportion of the appropriate structures affected in the entire section. Changes were graded for each component as follows: **Glomeruli**- size, lobulation, adhesions, fibrosis of the tufts, fibrosis of Bowman's capsule, dilatation, narrowing of capillaries, thickening of basement membranes, protein in Bowman's space, increased cellularity (mesangial or endothelial), infiltration by leukocytes, capillary thrombi. **Tubules**- atrophy, necrosis, vacuolar and hyaline droplet changes, basement membrane thickening, dilatation, inflammatory cells and casts in the lumen. **Interstitialium**- fibrosis, edema, acute and chronic leukocyte infiltration. **Arterioles**- fibrosis, thrombosis, hyaline change and narrowing. Analyses of blood urea nitrogen levels of 6 aged (10-13 months old) NOD-*scid* *IL2 $\gamma$* <sup>null</sup>*db/db* mice showed normal levels (23.7 mg/dl for NSG-*db/db* versus 22.8 mg/dl for NSG +/*db*). A separate cohort of mice is being aged for metabolic phenotyping at Vanderbilt University.

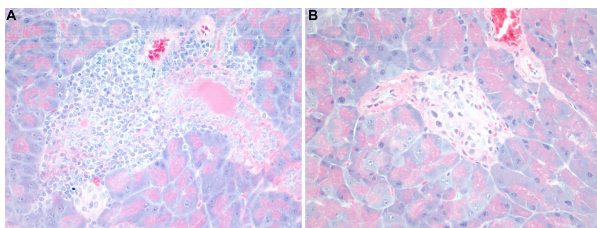
### Effect of NOD/Lt +/+ spleen cell transfer on pathological changes in NSG-*db/db* mice.

We tested the hypothesis that the absence of marked renal pathology in NSG-*db/db* mice was associated with the lack of a functional immune system. In order to reconstitute the immune system of NSG-*db/db* mice, ten million spleen cells from a 3 month-old female non-diabetic NOD/Lt +/+ mouse was injected intravenously into a group of 6 (7-10 week-old) NSG-*db/db* mice. Additional non-injected mice served as controls. The spleen cell recipients became moribund at 1 month post-injection and were found to be hyperglycemic (>600 mg/dl blood glucose). The mice were necropsied for histological analysis. As shown in **Figure 3**, kidneys from the NSG-*db/db* spleen cell recipients showed inflammatory changes including perivascular accumulation of lymphocytes.



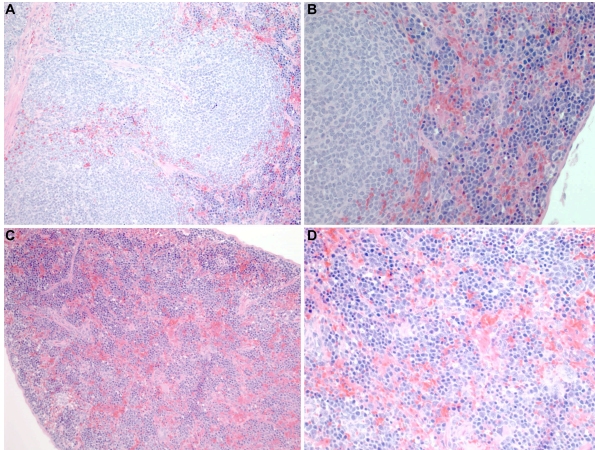
**Figure 3.** H&E stained kidneys sections from female NSG-*db/db* recipients of NOD/Lt +/+ spleen cells and a male uninjected control. A,B. NSG-*db/db* kidneys showing lymphocytic infiltration around blood vessels. C. Untreated NSG-*db/db* male showing normal structure. X 400.

It appeared likely that the hyperglycemia in the NSG-*db/db* spleen cell recipients was a consequence of the transfer of autoreactive diabetogenic T cells, the pancreata of these recipients were examined. As shown in **Figure 4**, insulinitis confirmed the transfer of autoimmune diabetes.



**Figure 4.** H&E stained pancreas sections from a female NSG-*db/db* recipient of NOD/Lt +/+ spleen cells and from a male uninjected control. A. NSG-*db/db* pancreas showing insulinitis. B. Untreated NSG-*db/db* male showing atrophied islet. X 400.

As shown in **Figure 5**, lymphocytic population of splenic follicles in the NSG-*db/db* recipients of NOD spleen cells confirmed the reconstitution of their immune system. The absence of follicles in untreated NSG-*db/db* mice confirms the absence of lymphocytes in this immunodeficient stock.



**Figure 5.** H&E stained spleen sections from a female NSG-*db/db* recipient of NOD/Lt *+/+* spleen cells and a male uninjected control. A,B. NSG-*db/db* spleen showing lymphoid follicles. C,D. Untreated NSG-*db/db* spleen showing absence of splenic follicles. A,C X 200; B,D x 400.

### Progress on Aim 2: Generation of NSG-*Nos3<sup>null</sup>* mice using a speed congenic approach

The *Nos3<sup>null</sup>* allele has been backcrossed to the NOD-*scid/scid IL2r $\gamma$ <sup>null</sup>* strain by marker assisted single nucleotide polymorphism (SNP) technology. We initiated the backcross by mating a C.ByJ.129P2(B6) *+/Nos3<sup>tm1Unc/J</sup>* (BALB/cByJ *+/Nos3<sup>null</sup>*) male heterozygote from The Jackson Laboratory repository to two NOD-*scid/scid IL2r $\gamma$ <sup>null</sup>* females. The (NOD x BALB/cBy) N1F1 *+/scid IL2r $\gamma$ <sup>null</sup>/Y +/Nos3<sup>null</sup>* male offspring from this cross were identified by PCR genotyping and backcrossed to NOD-*scid/scid IL2r $\gamma$ <sup>null</sup>* females. The PCR protocol to identify the *Nos3<sup>null</sup>* allele can be found on The Jackson Lab website: [http://jaxmice.jax.org/protocolsdb/f?p=116:2:1810697419322637::NO:2:P2\\_MASTER\\_PROTOCOL\\_ID,P2\\_JRS\\_CODE:3165,007073](http://jaxmice.jax.org/protocolsdb/f?p=116:2:1810697419322637::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:3165,007073). The N2 NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/Nos3<sup>null</sup>* males were typed for SNP markers across the genome and backcrossing continued using this speed congenic approach. We are now breeding the N3 generation (second round of speed congenics). We have had success in obtaining males with very high percentages of NSG genome in both rounds, with the current two breeding males having only 4 out of 109 markers still heterozygous for BALB/c SNPs. There will likely be offspring in the next generation (already born) that are 100% NSG based on SNP markers. They (the N4 generation) will be ready to send out for testing in June. NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/Nos3<sup>null</sup>* sibs will be intercrossed to produce NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> Nos3<sup>null</sup>* mice. The stock will be maintained by intercrossing NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/Nos3<sup>null</sup>* sibs. When developed, these mice will be characterized as carried out for NSG-*db/db* mice in Aim 1.

### RESEARCH DESIGN AND METHODS for work to be carried out with supplemental funding

#### Aim 3: Generation of NSG-*Nos3<sup>null</sup> db/db* mice by intercrossing NSG-*db/db* with NSG-*Nos3<sup>null</sup>* mice

We will intercross NSG-*db/db* and NSG-*Nos3<sup>null</sup>* mice to generate double mutant NSG-*Nos3<sup>null</sup> db/db* diabetic mice that should exhibit robust diabetic nephropathy symptoms on a stock of mice that can be engrafted with human cells and tested with human cell-based therapies for diabetic nephropathy. To develop the NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> db<sup>5J</sup>/db<sup>5J</sup> NOS3<sup>null</sup>* strain, NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/db<sup>5J</sup>* females will be mated to NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> Nos3<sup>null</sup>* males. The NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/db<sup>5J</sup> +/Nos3<sup>null</sup>* offspring will be genotyped by PCR and backcrossed to NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> Nos3<sup>null</sup>* mice. The NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/db<sup>5J</sup> Nos3<sup>null</sup>* offspring will be genotyped by PCR and intercrossed to produce NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> db<sup>5J</sup>/db<sup>5J</sup> Nos3<sup>null</sup>* mice along with NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/db<sup>5J</sup> Nos3<sup>null</sup>* controls. This colony will be maintained by matings of NOD-*scid/scid IL2r $\gamma$ <sup>null</sup> +/db<sup>5J</sup> Nos3<sup>null</sup>* sibs. When developed, these mice will be characterized as described for NSG-*db/db* and NSG-*Nos3<sup>null</sup>* mice in Aims 1 and 2.

**Potential Pitfalls:** We anticipate no problems with the generation of these stocks of mice as NSG-*db/db* mice are already available, and the backcross for the *Nos3<sup>null</sup>* mice to NSG has already been initiated in our laboratory. We recognize that we do not have a history in the study of diabetic complications, particularly in

the study of diabetic nephropathy. However, we have available the services of the NIH supported Mouse Metabolic Phenotyping Centers for the metabolic studies, and the services of Dr. Oded Foreman at The Jackson Laboratory, a board-certified veterinary pathologist, who will perform the histological analyses proposed. Also, by virtue of being part of the AMDCC, we will make these mice available to other members of the consortium who are experts in the study of diabetic complications for further phenotyping, histological, and metabolic analyses. Another caveat is that the background genome will affect the phenotype of *db/db* and *Nos3<sup>null</sup>* mice. We have already established the metabolic phenotype of the NSG-*db/db* stock, which is as predicted. However, we do not know if the NOD background is a susceptible or resistant strain for development of diabetic nephropathy. We note, however, that introduction of the *Nos3<sup>null</sup>* gene onto even the relatively nephropathy resistant C57BL/6 strain leads to symptoms of diabetic nephropathy in the presence of the *db/db* mutation.

**Significance:** By the completion of year 2 of the P&F, we will have 3 new stocks of immunodeficient mice with characteristics available for members of the AMDCC. First, we will have an immunodeficient stock of mice, the NSG-*db/db* strain, which will develop obesity, insulin resistance, and hyperglycemia with atrophic islets. Second, we will have available immunodeficient mice, the NSG-*Nos3<sup>null</sup>*, that should develop nephropathy in a normoglycemic environment. Third, we will have available NSG-*Nos3<sup>null</sup> db/db* mice that should develop diabetic nephropathy. These mice will provide models of individual components key for development of diabetic nephropathy and their combination in a single stock that will induce diabetic nephropathy in the absence of adaptive immunity. However, ***the key characteristic of the three stocks of mice that will be developed is that they can be engrafted with human cells and will provide a platform for the evaluation of human cellular therapy for the treatment of diabetic nephropathy.*** The direct *in vivo* evaluation of human cell therapies cannot be done in any other model system prior to phase I clinical trials where mechanistic analyses are extremely difficult to perform.