Animal Models of Diabetic Complications Consortium
(U01 DK076162)

Annual Report
(2008)

Diabetic Uropathy Pathobiology Site
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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypothesis- We hypothesize that depletion of manganese superoxide dismutase (MnSOD) specifically in smooth muscle of adult mice will exacerbate accumulation of reactive oxygen species (ROS) in smooth muscle during streptozotocin (STZ)-induced diabetes and accelerate the onset of the decompensation phase of diabetic bladder dysfunction. We further hypothesize that limiting depletion of MnSOD to arterial smooth muscle will have a lesser effect on STZ-induced diabetic bladder dysfunction by limiting exacerbation of STZ-induced ROS accumulation to the vasculature.

Recent Progress and Major Accomplishments:

Completion of breeding of healthy MnSOD$^{lox/lox}$ SM-CreER^{T2}(ki)$^{Cre/+}$ mice- We have completed the breeding of MnSOD$^{lox/lox}$ mice[1] with SM-CreER^{T2}(ki)$^{Cre/+}$ mice [2] and subsequently treated with OHT to activate CreER^{T2} to delete exon 3 of the MnSOD gene (SOD2) (Figure 1).

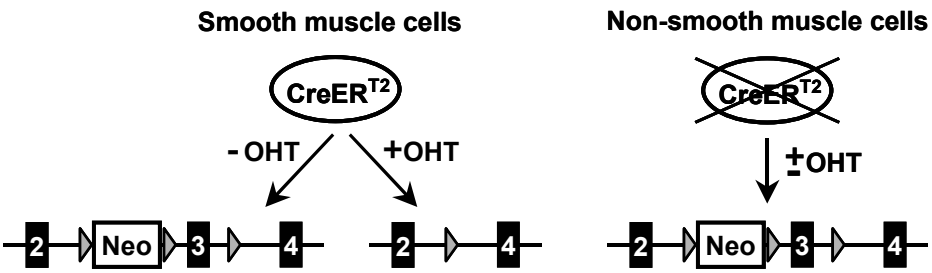

Figure 1. The strategy used for creation of our conditional, smooth muscle-specific MnSOD KO mice[2]. The lines at the bottom show the "floxed" MnSOD gene locus at exon 3. Exons 2-4, loxP sequences and the neomycin resistance gene are indicated by the numbered black boxes, grey triangles and boxed Neo, respectively. The circled CreER^{T2} indicates the CreER^{T2} protein.

In collaboration with Jackson Laboratory, we have established two colonies of this mouse model; one kept at JAX and the other in our laboratory. Currently, we are in the process of genotypical and phenotypical characterization of these mice. We expect that abolition of MnSOD will create an early decompensation of the bladder (earlier than 12 weeks) once the animals become diabetic with STZ injection.

Continuation of major progress on studies of pathophysiology of diabetic bladder dysfunction (DBD). Our lab continues to be at the forefront of examination of mechanisms of DBD. We have completed the following studies during 2007-early 2008:

Assessment of afferent autonomic function of the bladder in small animal models- Based on follow up on advise of EAC of AMDCC, and through a small grant support received (NICHD-R41-HD-04018: Transurethral Assessment of Altered Autonomic Function in Diabetic Bladder), we completed the development of a device (Bladder Sensory Threshold or BST) that can be implanted into the urinary bladder of rats[3] and mice (project supported by a supplemental grant from NIDDK Mouse Models and Phenotyping Consortium) and used with the Neurometer® CPT electrodiagnostic stimulator (Neurotron, Inc.) to assess afferent autonomic function of the bladder[3]. The Neurometer® is capable of delivering sine wave stimuli from 1 to 1000 μAmps at frequencies of 5, 250 or 2000 Hz that selectively stimulate small unmyelinated (C), small myelinated (Aδ) and large myelinated (Aβ) fibers, respectively. **Development of our device has been praised as a breakthrough that allows investigators to assess neuroselective disturbances of the afferent autonomic innervation of the bladder, which consists of C and Aδ fibers[4].** Our innovative work was published in the Journal of Urology[3], which included a 2 page editorial comment[4] on its current and future application. In which, Dr. Dale Bjorling

stated that 'the work present advancements in the search for improved methods to objectively assess bladder pain in their description of a technique for the measurement of bladder afferent nerve activity'. "The availability of objective data regarding relative activity of afferent fibers during conditions… could lead to a more precise understanding of mechanisms of pain sensation and improved care of patients."

Creation of a Urinary Diversion Model to examine the role of polyuria on DBD- We were the first group of investigators to demonstrate the temporal effects of T1D on bladder function in small rodents[5,6]. Our data revealed a striking adaptive change in the bladder from a hyperactive to an atonic state between 9 and 12 weeks after induction of DM by STZ, as indicated by a marked reduction in the peak voiding pressure (PVP) in both C57BL/6 mice[6] and Sprague Dawley rats[5]). For the most part, the temporal changes marking the early phase of DBD (<9 weeks in mice and rats) are also caused by osmotically-induced diuresis, pointing to polyuria and/or osmolality as potent causes of LUT hypertrophy in early phase of DBD. Following the above observation, we hypothesized that in the early stage of DM, osmotically-induced polyuria causes rapid hypertrophy and remodeling of the bladder, involving both neurogenic and myogenic components, leading to compensatory storage problems and hypertrophy-induced increased oxidative stress.

To examine the impact of polyuria on LUT, we have developed and maintained a model of urinary diversion (UD) in rats and characterized UD effects on the bladder and urethra in non-diabetic (completed experiments-data to be submitted) and diabetic (experiments in progress) animals. Our UD model is different from other attempted UD models, in that the ureters in our model are diverted to the cervix, which immediately drains the urine into the vagina (Figure 2. The epithelium of the vagina consists of keratinized squamous cells, similar to skin, with less permeability to urine compared to other choices of diversion such as colon or uterus. We have examined the functional (CMG), morphological and molecular profiles of the bladder and the urethra 1 week and 8 weeks after UD in 72 female Sprague-Dawley rats compared to normal and sham diversion controls.

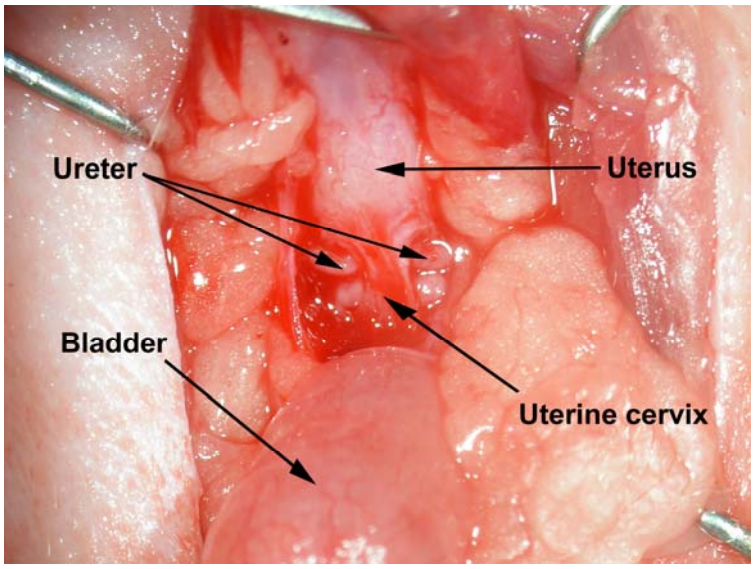

Figure 2. Urinary diversion in rat

Herein, we present the general, morphological and CMG data from our UD experiments:

Time point	Group	Body weight	Bladder weight	Bladder wt/ Body wt	Proportions of different bladder Tissues (% of total by Masson's stain, see Fig. 3)		
					Collagen	Muscle	Urothelium
1 week	control	240.7 ± 0.9	91.0 ± 2.7	0.38 ± 0.01	38.6 ± 1.2	48.4 ± 2.9	9.4 ± 0.2
	sham UD	247.5 ± 2.0	88.3 ± 2.0	0.36 ± 0.01	36.9 ± 2.5	49.9 ± 3.0	9.7 ± 0.7
	UD	242.7 ± 1.5	66.0 ± 2.2	0.27 ± 0.01	49.8 ± 1.9	34.6 ± 2.5	6.7 ± 0.6
8 weeks	control	280.9 ± 4.3	90.4 ± 1.8	0.32 ± 0.01	40.0 ± 2.0	42.7 ± 1.6	11.0 ± 0.5
	sham UD	281.5 ± 3.0	89.0 ± 2.1	0.32 ± 0.01	40.3 ± 1.7	44.4 ± 1.9	9.8 ± 0.7
	UD	277.3 ± 4.9	43.5 ± 2.4	0.16 ± 0.01	51.2 ± 1.4	39.0 ± 0.9	4.8 ± 0.6

ime poin	Group	**Basal Pressure** (cmH_2O)	**Threshold Pressure** (cmH_2O)	**Peak Pressure** (cmH_2O)	**Interval** (seconds)	**Voided volume** (ml)	**Compliance** (ml/cmH_2O)
1 week	control	12.15±0.62	13.71±0.24	58.96±9.06	437.7±51.7	0.56±0.08	0.40±0.052
	sham UD	12.14±2.71	14.13±2.90	60.23±17.86	554.5±29.5	0.76±0.06	0.39±0.057
	UD	14.76±1.61	32.49±0.75	52.10±1.80	143.3±24.5	0.20±0.02	0.01±0.002
8 weeks	control	16.72±2.21	19.28±1.34	59.18±4.92	542.3±69.3	0.78±0.13	0.36±0.081
	sham UD	281.5 ± 3.0	17.92±1.81	63.67±3.11	574.2±26.7	0.76±0.04	0.40±0.07
	UD	16.43±4.19	18.92±3.66	58.45±9.75	118.5±22.8	0.14±0.02	0.07±0.010

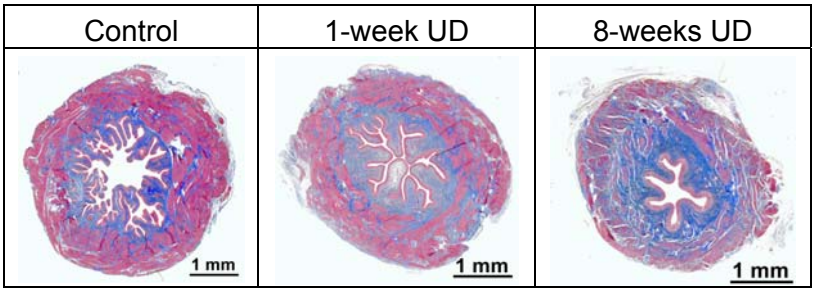

Figure 3. Representative images of Masson's trichrome staining of equatorial sections of urinary bladders from control, 1-week UD and 8-week UD rats, showing smooth muscle (outer magenta), collagen (blue) and urothelium (inner light magenta).

External Urethral Sphincter Activity in Diabetic Rats[7]. The objective our study was to examines the temporal effects of diabetes mellitus (DM) on the bladder and the external urethral sphincter (EUS) activity in diabetic rats. In this study, 24 female Sprague Dawley (SD) rats were divided into 2 groups: streptozotocin-induced diabetics and age, sex-matched controls. Cystometry (CMG) under urethane anesthesia and electromyogram (EMG) of the EUS were evaluated in all rats after 6 and 20 week of diabetes induction. After EMG assessment, the tissues of the-urethra were harvested for morphological examination. Our results showed that diabetes caused reduction of body weight compared to controls, and the bladders of diabetic rats weighed more than the controls after 6- or 20-weeks of diabetes induction. CMG measurements showed diabetes increase threshold volume, contraction duration, high-frequency oscillations (HFO), and higher residue volume. Peak contraction amplitude increased in 6-week but not in 20-week diabetic rats. EUS-EMG measurements showed significantly increased frequency of EUS-EMG bursting discharge during voiding in 6-week (8.1±0.2, 6.9±0.6/sec, respectively) but not in 20-week (5.8±0.3, 6.0±0.2/sec, respectively) diabetic rats compared to controls. EUS-EMG bursting period was also increased in 6-week (6.8±0.3,

4.1±0.6 sec, respectively) and 20-week (7.5±0.6, 4.3±0.4 sec, respectively) in diabetic rats compared to controls. EUS-EMG silent periods were reduced in 6-week (0.072±0.004, 0.100±0.010 sec, respectively), not changed in 20-week (0.135±0.015, 0.115±0.005 sec respectively), but active period did not change in 20-week, increased in 6-week diabetic rats compared to controls. Morphometric analysis showed atrophy of striated muscle in the EUS after 20 week but not 6 week of DM induction. From this study we concluded that diabetes causes marked functional and anatomical abnormalities of the EUS. These abnormalities may contribute to the previously reported time-dependent bladder dysfunction in diabetic rats.

Assessment of bladder function in T2D mice. (Data not shown) We have recently completed pilot studies of LUT dysfunction in monogenic mice models of T2D and obesity in relation to our work within the AMDCC and related to studies of animal models of urinary incontinence[8]. The studies have included 24 hr micturition habits, CMG, measurements of leak point pressure (LPP). The models have included C57Bl6/db/db and C57Bl6/ob/ob mice with their respective age and sex-matched controls. The initial aim of these experiments was to assess the presence or absence of urinary incontinence in these animals as measured by LPP. Further, the animals underwent survival surgery (vaginal distension and implantation of suprapubic tube) for 20 days[9].

Intra-bladder neurofilaments (unpublished data). To assess the impact of diabetes on neurogenic elements of the LUT, we detected and quantified the alterations in intra-bladder neurofilaments in equatorial sections of urinary bladders by immunofluorescence as previously described[9]. The preliminary results indicated that the density of intra-bladder neurofilament-immunoreactive nerves significantly decreases 20 weeks after induction of DM or diuresis compared to control, but is more pronounced in diabetes (Fig.).

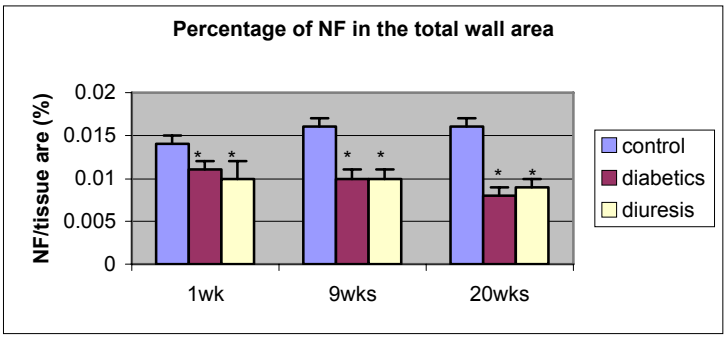

Figure - Quantification of neurofilament in the bladder indicates marked and progressive decrease in diabetes and diuresis. After 20 weeks, diabetes causes more loss of neurofilaments than diuresis.

Specificity of spinal c-Fos expression induced by Neurometer®-bladder CPT electrostimulation (submitted: Journal of Urology- Data not shown). The neuroselectivity of Neurometer® electrostimulation of bladder afferent pathways was assessed using expression in different spinal cord regions of the protooncogene c-Fos, known to be induced by increased neuronal activity, as a marker. Using the Neurometer® with our newly developed BST device, sine-wave electrical stimulation was applied for 90 minutes to the bladder in rats. Following Neurometer® stimulation at 5 Hz with a current of 2.0 mA, the distribution of immunocytochemically-detected c-Fos positive cells in the spinal cord segments (L1 to S1) that contribute axons to the pelvic and hypogastric nerves was measured (Fig. 2). The distribution of a major peak of c-Fos expression in L6 and minor peaks in L1 and S1 was very similar to that found in rats that received a 30 minute intravesical injection of capsaicin instead of Neurometer® stimulation. Since capsaicin stimulates predominantly C-fibers that experiment provides evidence for C-fiber selectivity of Neurometer® stimulation at 5 Hz.

Plans for the Upcoming Year- our plans for the next year are along two parallel pathways: a) continue our investigation of pathophysiology of diabetic bladder dysfunction; b) phenotype and genotype characterization of our created $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}$ mice, begin the experiments related to the following specific aims. For all the experimental studies, we will use the following groups of mice:

1. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ treated with OHT to activate $CreER^{T2}$ to abolish MnSOD expression.
2. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ treated with OHT and with STZ to induce diabetes.
3. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$, sham treated.
4. $MnSOD^{lox/lox}$, $SM\text{-}CreER^{T2}(ki)^{Cre/+}$ treated with STZ.

Specific aim #1: To examine the temporal alterations in the in-vivo bladder function by evaluation of 24 hours micturition habits and conscious cystometry in the above groups of mice at two time points of 8 and 12 weeks after induction of diabetes.

Specific aim #2: To examine the temporal course of morphological changes in neurogenic and myogenic components of the bladder remodeling in the above groups of mice by:

1. Examination the changes of bladder tissue components and their contribution to remodeling of the wall and chamber of the bladder
2. Examination of the changes in bladder innervations markers.

We anticipate that the following experiments will be done after 2008:

Specific aim #3: To examine the temporal alterations in the contractile function of the detrusor in the above groups of mice by:

1. Examination of the contractile responses of the detrusor.
2. Examination of the contractile and regulatory proteins of the detrusor.
3. Examining the alterations of the L-type Ca^{2+} channel.
4. Examining the alterations in the capacitive calcium entry (CCE).
5. Examining the IP3- and RyR-induced calcium release.
6. Examining the Ca^{2+} sensitivity in permeabilized detrusor strips.

Specific aim #4: To examine the temporal alterations induced by STZ in afferent and efferent autonomic pathways innervating the bladder in the in the above groups of mice by:

1. Assessment of afferent autonomic function by measurement of Current Perception Threshold (CPT)
2. Examining the relative contribution of cholinergic and purinergic components to the contractile response to transmural electrical stimulation.
3. Examining the alterations in ATP-P2X3, VR-1 afferent pathway in the bladder.
4. Examining the alterations in muscarinic receptors (M2, M3) and/or purinergic receptors (P2X1, P2X2).
5. Examining the connexin 43-containing gap junctions in the bladder.

Preliminary Milestones for 2009 and Beyond- With moving along our experiments, and availability of the created mice from other sites of AMDCC, we will begin phenotyping of some of the mice created by other groups to examine the presence of diabetic uropathy in those mice. We would particularly be interested in mice models of neuropathy.

Collaboration:

With other AMDCC PIs- We obtained the MnSOD$^{lox/lox}$ from the laboratory of Dr. Frank Brosius at the University of Michigan.

With Jax- In collaboration with Dr. Leiter at Jax we have completed and maintained two colonies of MnSOD$^{lox/lox}$ SM-CreER^{T2}(ki)$^{Cre/+}$ mice at Jackson lab and our laboratory.

With the MMPCs- We responded to the MMPC internal RFA for development of Bladder Sensory perception Threshold (BST) device in mice and received approval and funding. The BST device would allow us to assess the bladder afferent sensory function as a phenotyping measure in diabetic mice. We are awaiting the results of the review of the RFA.

With other non-AMDCC PIs- We continue to have active collaboration with internal (Cleveland Clinic) and external investigators in the Cleveland Area. The followings are some of our active collaborators:

1. Margot Damaser, Ph.D.- Lerner Research Institute (LRI) of the Cleveland Clinic- Dept of BME-we have the most extensive collaboration with Dr. Damaser's research team. Our collaboration extents from sharing joint lab space, joint experiments, joint mentoring of trainees; joint weekly lab meetings; and submission of several research proposals.
2. Timothy Kern, Ph.D.- Case- Department of Medicine and Ophthalmology- We have extensive collaboration with Dr. Kern extending from sharing animals for joint experiments to monthly joint lab meetings that are alternatively held at Case or CCF campus.
3. Vincent Monnier, M.D.- Case- Department of Pathology- to study the role of Advanced Glycation Endproduct in Diabetic Bladder Dysfunction.
4. Manju Bhat, Ph.D.- LRI- Center for Anesthesia Research- to study the mechanisms of calcium influx into the neuron and detrusor muscle cells.
5. Fernando Casas, Ph.D.- LRI- BME- to study the integration of vocalization of animal models into the assessment of afferent function of the bladder.
6. Lori Birder, Ph.D. and Anthony Kanai, Ph.D. from Departments of Medicine and Pharmacology of the University of Pittsburgh- Our collaboration stated from studies of role of urothelium and reactive oxidative stress products in mechanisms of diabetic bladder dysfunction and led to our joint project funded by JDRF for 2006-2008.
7. Stanley Hazen, M.D. Ph.D.- LRI- to study the role of oxidative stress in Diabetic Bladder Dysfunction
8. Jianguo Cheng, M.D. Ph.D.- Department of Anesthesia and Pain Management- to study the innervation of the lower urinary tract.

Address previous EAC comments:

a. Can you reverse bladder decompensation with insulin?

Perhaps yes. Our previous experience (REF) shows that the treated DM animals behave similar to those of controls at least up to 20 weeks of diabetes, if the treatment starts immediately following establishment of hyperglycemia. Since the bladder remodeling begins as early as 4 days after establishment of hyperglycemia (blood sugar of 300 mg/dl) which is accompanied by hyperosmolar polyuria, the reversal of decompensation could only be delayed and not prevented. To find answer to a part of this question, we have developed the urinary diversion

model to devoid the bladder from early changes. We will report the results in our next year's annual report.

b. Continued efforts must be made to establish validation criteria for diabetic uropathy (decreased bladder sensation, increased peak pressure reversible with glu control, histopathology such as increased SM, decreased nerve fiber density, etc.).

We could not have agreed more. A major portion of our work is related to such validation criteria as indicated by our publication list.

c. One of the highlights of the consortium. Dr. Daneshgari continues to produce pioneering work in this field (like the BST measurements).

On behalf of our hard working research team, I thank you for your kind words.

d. Publications:

1. Lee UJ, Gustilo-Ashby AM, **Daneshgari F,** Kuang M, Vurbic D, Lin DL, Flask CA, Li T, Damaser MS: Lower Urogenital Tract Anatomical and Functional Phenotype in Lysyl Oxidase Like-1 Knockout Mice Resembles Female Pelvic Floor Dysfunction in Humans. Am J Physiol Renal Physiol. 2008 May 21. [Epub ahead of print]
2. Hijaz A, **Daneshgari F**, Sievert KD, Damaser MS: Animal models of female stress urinary incontinence; A review Journal of Urology 2008 Jun;179(6):2103-10. Epub 2008 Apr 18
3. Guiming Liu, Mei Li, **Firouz Daneshgari**: Calcineurin but not Akt signaling is involved in remodeling of the bladder detrusor muscle in diabetic rat. Am J Physiol Regul Integr Comp Physiol 2008 (in press)
4. Abouassaly R, Liu G, Yamada Y, Ukimura O, **Daneshgari F:** Efficacy of a novel device for assessment of afferent autonomic sensory function in the rat bladder. Journal of Urology 2008 Mar;179(3):1167-72
5. Liu G, Lin YH, Yamada Y, **Daneshgari F**: External Urethral Sphincter Activity in Female Diabetic Rats. Neurourology and Urodynamics 2008 March 19 (Epub)
6. Kim JH, Huang X, Liu G, Moore CK, Bena J, Damaser MS, **Daneshgari F**. Diabetes slows the recovery from urinary incontinence due to simulated childbirth in female rats. Am J Physiol Regul Integr Comp Physiol. 2007 May 9
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8. Lin YH, Liu G, **Daneshgari F:** A mouse model of simulated birth trauma induced stress urinary incontinence. Accepted for publication at Neurourology & Urodynamics July 2007
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Respectfully Submitted,

Firouz Daneshgari, M.D.

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3. Abouassaly R, Liu G, Yamada Y, Ukimura O and Daneshgari F: Efficacy of a novel device for assessment of autonomic sensory function in the rat bladder. J Urol 2008; **179:** 1167.

4. Bjorling DE: Measuring bladder pain. J Urol 2008; **179:** 815.

5. Daneshgari F, Liu G and Imrey PB: Time dependent changes in diabetic cystopathy in rats include compensated and decompensated bladder function. J Urol 2006; **176:** 380.

6. Daneshgari F, Huang X, Liu G, Bena J, Saffore L and Powell CT: Temporal differences in bladder dysfunction caused by diabetes, diuresis, and treated diabetes in mice. Am J Physiol Regul Integr Comp Physiol 2006; **290:** R1728.

7. Liu G, Lin Y, Yamada Y and Daneshgari F: External urethral sphincter activity in diabetic rats. Neurourol Urodynam 2008; **Published online ahead of print:**

8. Hijaz A, Daneshgari F, Sievert KD and Damaser MS: Animal models of female stress urinary incontinence. J Urol 2008; **179:** 2103.

9. Lin YH, Liu G, Li M, Kavran M and Daneshgari F: Temporal effects of vaginal distension and bilateral pudendal nerve transaction on leak-point pressure and urethral anatomy in female mice. Neurourol Urodyn 2008; **27:** 106.