Diabetic Complications Consortium

Application Title: The Influence of Esm-1 on Leukocyte Infiltration in Diabetic Nephropathy

Principal Investigator: Vivek Bhalla, MD, FASN

1. Project Accomplishments:

With the support of the DCC, we have been able to make substantial progress on this project.

For Aim 1, we have initiated a collaboration with biophysicist, Mohammad Kiani at Temple University to show for the first time that Esm-1 inhibits leukocyte infiltration *in vitro* in a dose-dependent manner.

For Aim 2, we have discovered several genes with differential expression in glomeruli from mice susceptibility or resistance to diabetic nephropathy. We have validated these genes using qPCR, and have further validated Esm-1 at different time points after the onset of diabetes. Furthermore, we optimized a glomerular culture *ex vivo* to measure levels of secreted protein, Esm-1 in conditioned media at 4 and 10 weeks duration of diabetes.

We have also investigated the levels of Esm-1 in glomeruli with and without hyperglycemia, and have studied the effect of diabetes on serum and urine Esm-1.

For Aim 3, we have working toward over-expressing high circulating levels of Esm-1. Interestingly, we have Esm-1 levels increase only transiently, and then quickly decrease, unlike other cytokines given similarly. Based on some *in vitro* experiments, we think this may indicate that Esm-1 is regulated by proteolysis (data not shown). To accomplish our goal put forth in Aim 3, we have begun an alternate strategy to make the knockout of Esm-1 using a tamoxifen-inducible endothelial-cell specific Cre and floxed Esm-1 embryos.

We have learned that Esm-1 is functionally relevant for leukocyte infiltration; that it is expressed in inverse relation to the susceptibility of diabetic nephropathy; and is dynamically regulated in the whole animal and in the kidney with diabetes.

I would like to thank the members of the study section for the Pilot & Feasibility grant, and also the DCC Steering Committee membership as these funds have provided data that will enhance the field. We are preparing for submission of a manuscript as well and have provided preliminary data for grant applications that we have submitted. This data has already been used by my postdoctoral fellow, Xiaoyi Zheng, to obtain a prestigious 3-year Larry L. Hillblom Foundation postdoctoral award. I have also applied for grants to the American Diabetes Association and plan to apply to others including the DCC Pilot&Feasibility 2015.

2. Specific Aims:

Aim 1. Perform a dose-response curve for Esm-1 efficacy to inhibit leukocyte adhesion to glomerular endothelial cells *in vitro* and *in vivo*.

Results:

Using a novel flow chamber assay, Esm-1 dramatically decreases leukocyte rolling and transmigration across an endothelial cell monolayer. Esm-1 has been demonstrated to disrupt LFA-1:ICAM-1 interaction, and is thus, predicted to inhibit leukocyte transmigration. We tested the functional role of Esm-1 in collaboration with Dr. Mohammad Kiani (Mechanical Engineering, Temple University) by exposing leukocytes to Esm-1 vs. vehicle and measuring leukocyte rolling, adhesion, and transmigration (**Figure 1**) using a bio-inspired microfluidic device as a flow chamber assay to study leukocyte-endothelial cell interactions on a chip[2]. We demonstrate a 73% inhibition of leukocyte rolling, no effect on adhesion, but surprisingly a persistent, and substantial, 71-75% reduction in migration from 10 to 60 minutes. We have also repeated these experiments with lower doses, predicted to be the local concentration of Esm-1, based on glomerular secretion assays (**Figure 2**). These preliminary results provide strong evidence to pursue *in vivo* experiments related to Esm-1 as a protective agent against leukocyte infiltration, and precede the proposed *in vitro* experiments to perform a dose titration; to test the role of Esm-1 to prevent different subtypes of leukocytes; and to confirm this inhibition using murine glomerular endothelium from the different strains of mice (e.g. rescue using Esm-1 deficient endothelial cells from DN-susceptible DBA/2J mice).

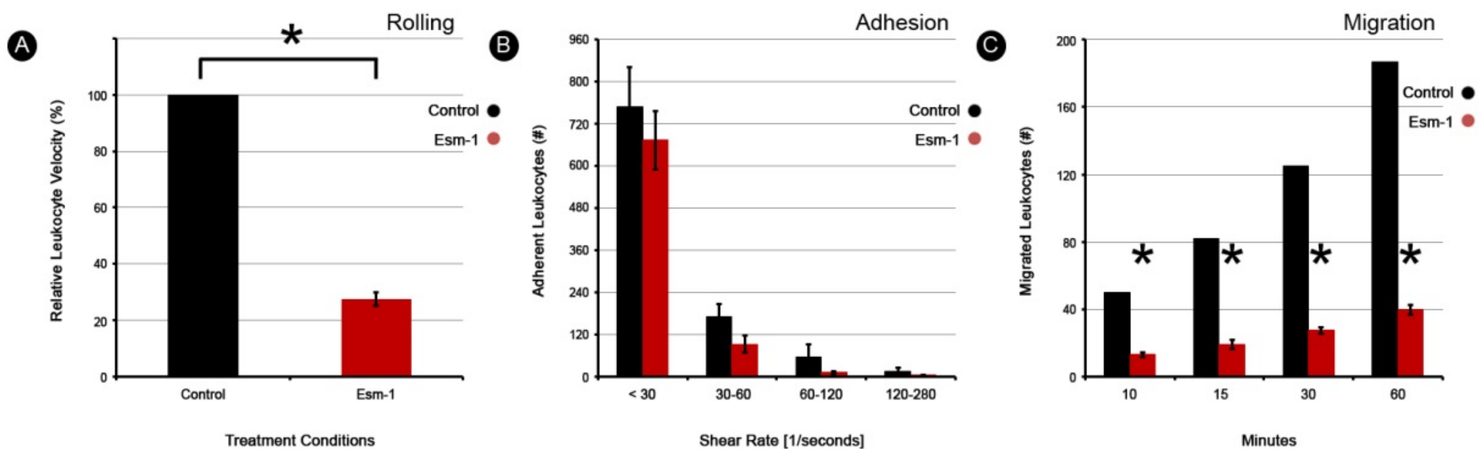

Figure 1: Esm-1 decreases leukocyte rolling and transmigration across endothelium *in vitro*. Vascular channels derived from mouse cremasteric capillary network were etched onto a chip and coated with fibronectin. Endothelial cells (HUVECs)(2*10^7 cells/mL) were seeded and channels were under maintained in a flow chamber overnight. Confluent endothelial cells were activated for 4 hours with 10 ng/mL TNF-α. Human neutrophils were isolated, treated with vehicle (black bars) vs. 500 ng/mL of Esm-1 (red bars) for 60 minutes at 4C, labeled with CFDA, and activated with TNF-α. A chemoattractant fMLP[2] (1μM) was injected into the tissue area, followed by perfusion of channels by activated neutrophils. Rolling, adhesion, and migration of leukocytes were quantified as detailed in Lamberti, et al[2]. (A) Esm-1 significantly decreased rolling velocity by 73% compared with control (vehicle)-treated leukocytes. (B) The number of adherent leukocytes was not significantly different between control and Esm-1. (C) The number of migrating leukocytes was significantly diminished between 71-75% at 10-60 minutes. Results are shown as average ± SEM. *, $p<0.05$. N=3 experiments with Esm-1 and N=2 with vehicle.

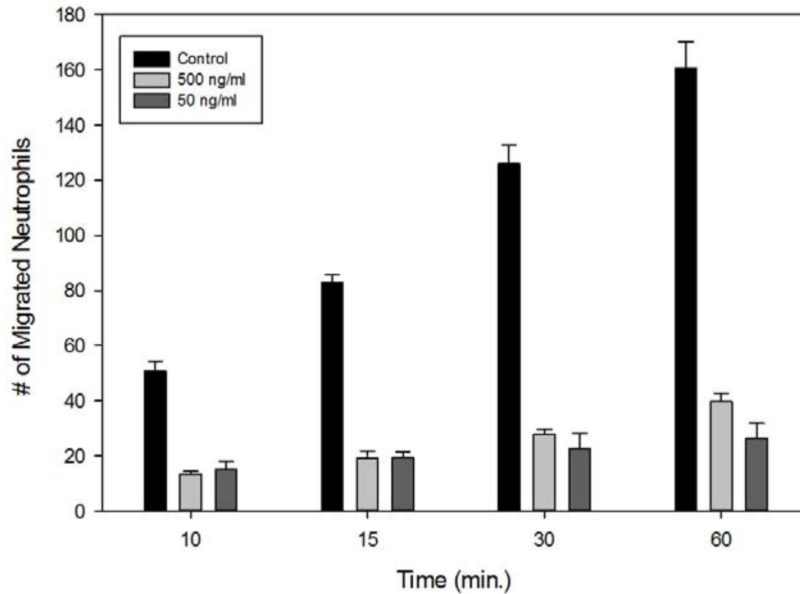

Figure 2: Esm-1 decreases transmigration across endothelium *in vitro* in a dose-dependent manner. Vascular channels derived from mouse cremasteric capillary network were etched onto a chip and coated with fibronectin. Endothelial cells (HUVECs)($2*10^7$ cells/mL) were seeded and channels were under maintained in a flow chamber overnight. Confluent endothelial cells were activated for 4 hours with 10 ng/mL TNF-α. Human neutrophils were isolated, treated with vehicle (black bars) vs. 50 or 500 ng/mL of Esm-1, as indicated (*light* and *dark gray* bars, respectively) for 60 minutes at 4C, labeled with CFDA, and activated with TNF-α. A chemoattractant fMLP[2] (1μM) was injected into the tissue area, followed by perfusion of channels by activated neutrophils. Migration of leukocytes were quantified as detailed in Lamberti, et al[2]. Results are shown as average ± SEM. *, $p<0.05$. N=3 experiments.

Aim 2. To determine the optimal timing for therapeutic Esm-1 delivery, evaluate Esm-1 expression in glomeruli from DN-susceptible and DN-resistant mice.

Results:

Glomerular expression patterns differ between DN-susceptible and DN-resistant mice. To better understand the signals which mediate progression of DN, we have compared glomerular gene expression profiles from different strains of mice either susceptible or resistant to DN (DBA/2J vs. C57BL/6J mice induced with low-dose streptozotocin, respectively)[10, 12] (**Figure 3**). In microarray experiments from mice harvested at an early time point before evidence of DN on histology (i.e. 4 weeks after induction of diabetes mellitus) (**Figure 4**), we have demonstrated that when we confined our analysis to those genes which were only significantly different in samples from diabetic mice, 22 genes are up-regulated greater than 2-fold, while 8 genes are down-regulated in glomeruli from mice susceptible to DN when adjusted for multiple comparisons. We validated several up- and down-regulated genes by qPCR and among these we selected Esm-1, Tsc22d3, and Midkine for further characterization based on a putative common mechanism of action.

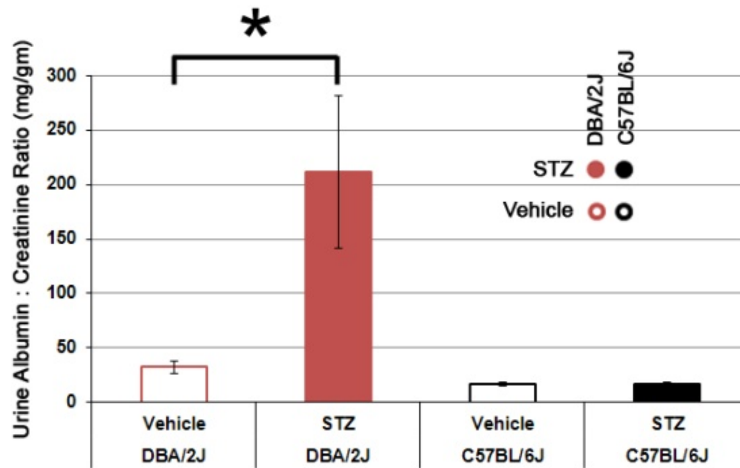

Figure 3: Confirmation of differential susceptibility to clinical DN in different strains of mice. DBA/2J (red) and C57BL/6J (black) male mice were induced with low-dose streptozotocin (STZ) vs. vehicle (45 mg/kg) daily for 5 days at week 0, and assayed urine albumin : creatinine ratio at week 16 after onset of diabetes mellitus. Diabetic DBA/2J mice (DN-susceptible) exhibit significant STZ-dependent albuminuria compared with C57BL/6J mice (DN-resistant). *, $p < 0.01$, N=4-6 mice / group.

Glomeruli from DN-susceptible mice are deficient in Esm-1 and Tsc22d3 and produce high Midkine levels relative to DN-resistant mice. We discovered that Esm-1 was significantly down-regulated by 3.3x-fold in DN-susceptible vs. DN-resistant mice by microarray. Furthermore, upon validation with qPCR, Esm-1 was decreased with induction of diabetes in DN-susceptible mice, and increased with induction of diabetes in DN-resistant mice. In total, Esm-1 expression was ~15x-fold higher levels in DN-resistant mice despite no significant changes in expression of known inducers / repressors of Esm-1 expression (e.g. VEGF-A/C, Hhex)[39, 40] (data not shown). Additionally, we found that Tsc22d3 expression was ~2x-fold lower and Midkine expression was ~6x-fold higher in DN-susceptible mice. These results in conjunction with the existing literature suggest that Esm-1 and Tsc22d3 may be protective genes, and Midkine may promote disease (**Figure 5**).

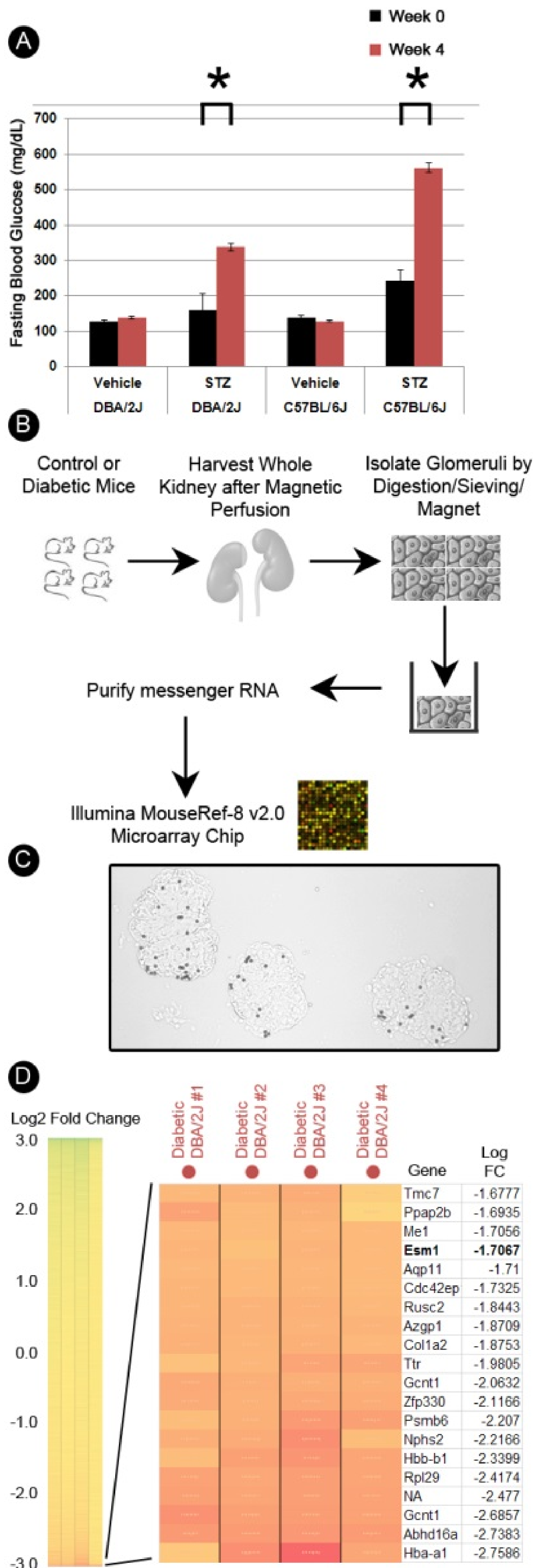

Figure 4: Comparison of glomerular gene expression in diabetes between different strains of mice. DN-susceptible (DBA/2J, red) or DN-resistant (C57BL/6, black) male mice were induced with low-dose streptozotocin (STZ) vs. vehicle (45 mg/kg) daily for 5 days at week 0, N=4 mice / group. (A) Fasting blood glucose was measured at week 4 after induction with STZ vs. vehicle. *, $p < 0.05$, N=4 mice / group. (B) Glomeruli from these four groups of mice were harvested after vascular perfusion with magnetic beads. Whole kidney was minced and digested, and glomeruli were obtained by sequential sieving followed by magnetic isolation. (C) Glomerular preparations were examined under low-power magnification to confirm isolation of glomeruli. After we measured RNA quality and purity, we collaborated with the Stanford Functional Genomics Facility to label RNA and hybridize to an Illumina array. Sixteen samples were analyzed (N=4 mice / group) for differential gene expression. Expression patterns were normalized using quantile normalization in R statistical software and differences were deemed significant if >= 2-fold between groups and if the p-value (adjusted for multiple comparisons using Benjamini–Hochberg) was < 0.05. (D) A Heat-map of normalized signal intensity is shown demonstrating the range of differential log base 2 fold change (Log FC) in glomerular gene expression between diabetic DBA/2J and the average signal from diabetic C57BL/6 mice as the reference group. Each column represents an individual diabetic DBA/2J mouse signal vs. the reference signal. *Inset,* genes with lowest expression (red) in DBA/2J vs. C57BL/6J samples, e.g. including Esm-1 (bold). (E) From the microarray results, we narrowed the list to genes that were significantly different between strains among only diabetic mice but were unchanged in non-diabetic mice from the two strains. The list of 30 significantly up- (green) and down-regulated (red) genes and their average fold change from microarray results. Esm-1 and Tsc22d3 expression are significantly lower, while Midkine expression is higher, in DN-susceptible DBA/2J vs. DN-resistant C57BL/6J mice (selected genes are shown in bold).

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Gene Name	Fold Change	Gene Name	Fold Change
Mdk	5.64	Oas1g	2.19
Cyp4a12a	4.22	Prcp	2.19
Cyp4a12b	3.28	Kynu	2.19
Sectm1b	2.90	Ugt1a6a	2.13
Hsd3b2	2.89	Ccbl2	2.08
Slc7a7	2.60	Apol9b	2.06
Tmem82	2.41	Gabrb3	2.05
Lyplal1	2.35	1300014I06Rik	0.50
Fam20b	2.32	Csrnp1	0.44
Irgm1	2.32	**Tsc22d3**	0.41
Adi1	2.29	Hmgn3	0.41
Plau	2.26	Rasl11b	0.38
C3	2.26	Cd300lg	0.36
Pde1a	2.22	**Esm1**	0.31
Dpp7	2.22	Sspn	0.29

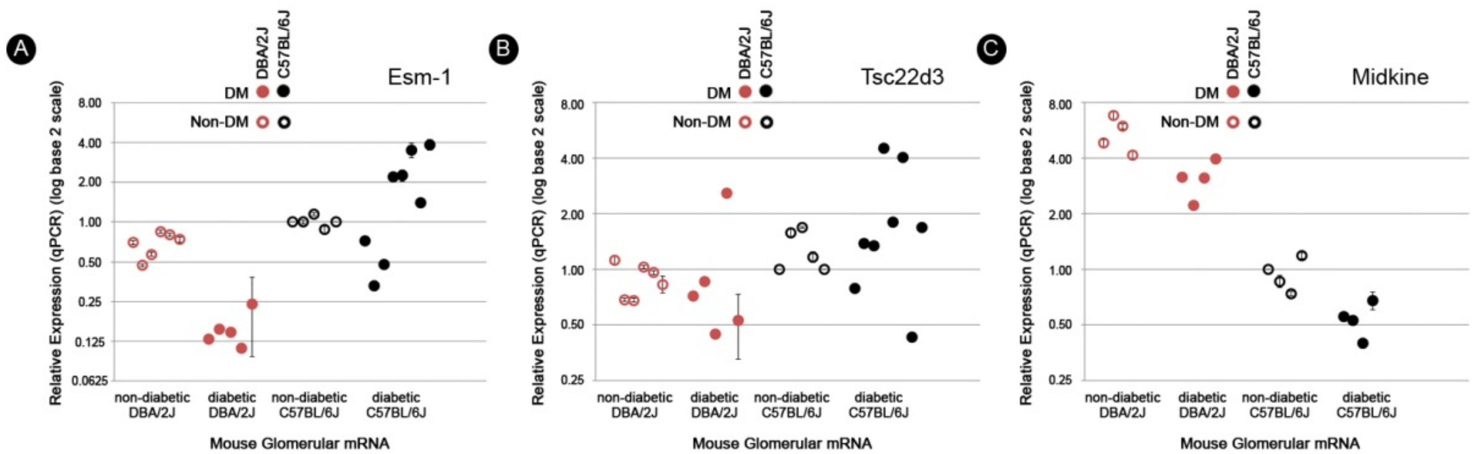
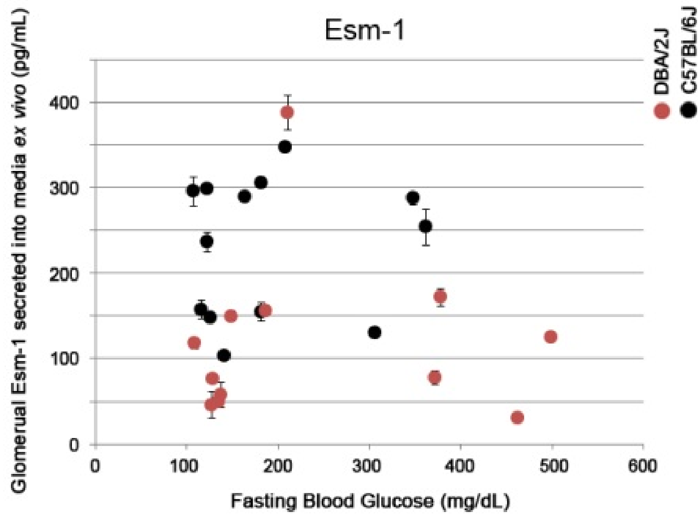


Figure 5: Validation of microarray results for Esm-1, Tsc22d3, and Midkine. By quantitative PCR (qPCR) we measured glomerular Esm-1, Tsc22d3, and Midkine expression in separate cohorts of mice. (A) Esm-1, levels were significantly ~15x-fold higher in diabetic DN-resistant (C57BL/6J, black solid circles) relative to diabetic DN-susceptible (DBA/2J, red solid circles) mice. Alteration in Esm-1 upon induction of diabetes was opposite in the two strains. Esm-1 was decreased in DN-susceptible mice, and increased in DN-resistant diabetic mice. (B) Tsc22d3 levels were significantly lower in diabetic DBA/2J (red solid circles) vs. diabetic C57BL/6J (black solid circles) mice. (C) Midkine levels were significantly higher in diabetic DBA/2J (red solid circles) vs. diabetic C57BL/6J (black solid circles) mice. qPCR data is expressed on a log-base 2 scale relative to GAPDH and relative to non-diabetic C57BL/6J (black hollow circles) as a referent group. Each circle represents data from an individual mouse and error bars indicate standard deviation of replicates in the qPCR assay. Differences were determined to be significant if $p<0.05$, N=5-8 mice/group. DN-susceptible (DBA/2J, red); DN-resistant (C57BL/6, black); Hollow circles (non-diabetic); solid circles (diabetic) mice.

Figure 6: Glomeruli from DN-susceptible mice exhibit Esm-1 deficiency. Glomeruli from diabetic and non-diabetic DN-susceptible (DBA/2J, red) mice and DN-resistant (C57BL/6J, black) mice were isolated from kidney and cultured *en bloc* in DMEM with 0.2% FCS overnight. The amount of Esm-1 secreted (pg/mL) into the culture media was measured by ELISA. Glomeruli from DN-susceptible mice secrete significantly less Esm-1. The results from each mouse are plotted against the fasting blood glucose (mg/dL) at the time of sacrifice. The cultured media of 293T cells transfected with mouse Esm-1 and recombinant Esm-1 protein were positive controls. Non-cultured media and mock-transfected 293T cell cultured media were negative controls (not shown). Each point represents data from an individual mouse. Error bars are from replicated wells in ELISA. p**< 0.01, N=11-13 mice / group.**

To further validate the differential expression of our gene products, we quantitated Esm-1 protein. We were unable to detect intracellular Esm-1 protein by western blot or immunohistochemistry. Alternatively, from these two strains of mice, we briefly cultured isolated glomeruli *ex vivo* and quantitated the secreted (active) form of the protein by a commercial ELISA kit (Aviscera Bioscience). DN-susceptible mice secrete approximately 50% less Esm-1 than DN-resistant mice (120.5+/-27.9 vs. 231.5+/-22.6, $p<0.01$, N=11-13 mice/group) (**Figure 6**).



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We have also repeated this experiment, at 10 weeks of diabetes (**Figure 7**).

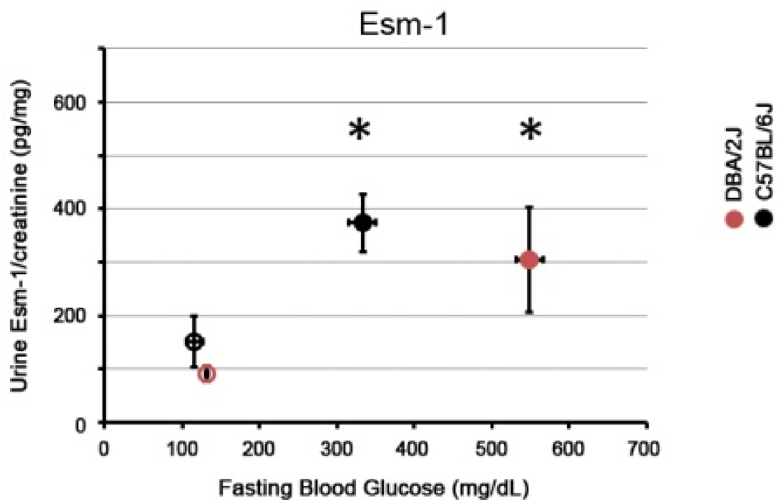

Figure 7: Glomeruli from DN-susceptible mice exhibit Esm-1 deficiency at 10 weeks of diabetes. *, p-value < 0.05 vs. non-diabetic.

We have also studied the levels of Esm-1 in serum (**Figure 8**) and urine (**Figure 9**) with and without diabetes. With an STZ model, Esm-1 decreases in serum and increases in urine. Esm-1 also increases in the conditioned media of glomeruli with hyperglycemia (**Figure 10**), so we believe the increase in urine is emanating from glomerular Esm-1. Albuminuria is unchanged under these conditions, so it is less likely due to increase filtration (data not shown).

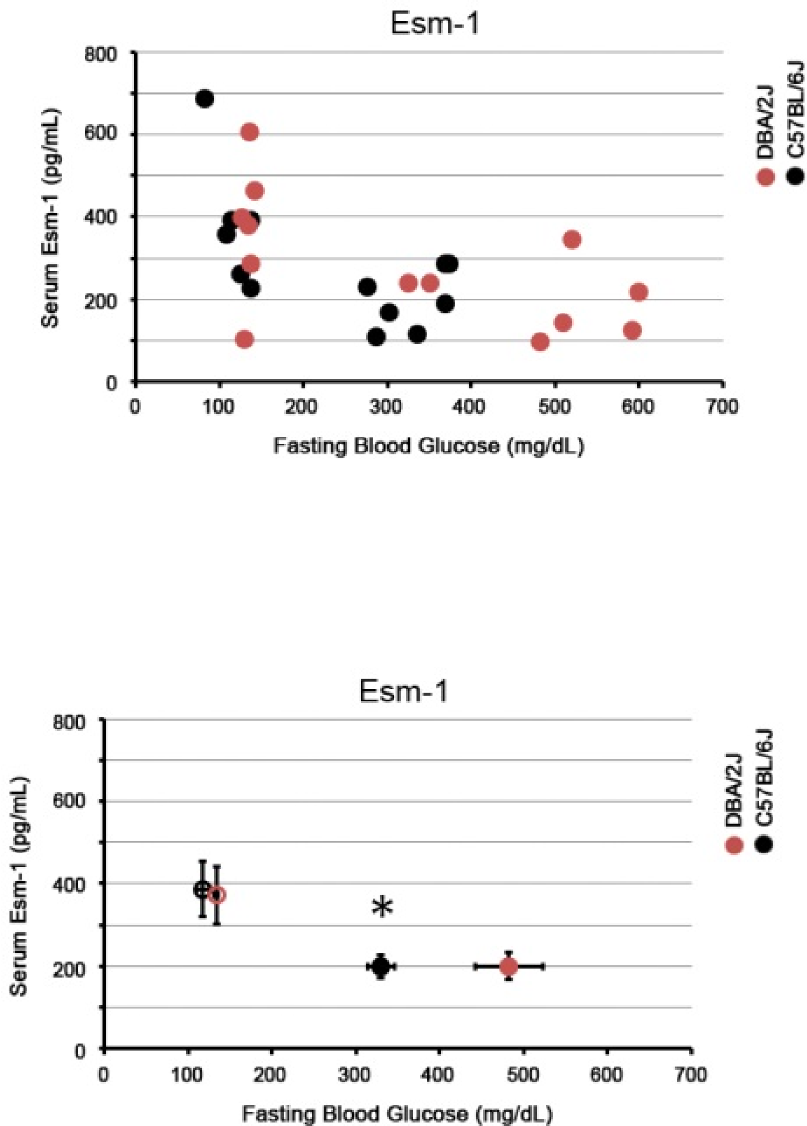

Figure 8: Esm-1 in serum with 4 weeks of diabetes. *, p-value < 0.05 vs. non-diabetic.

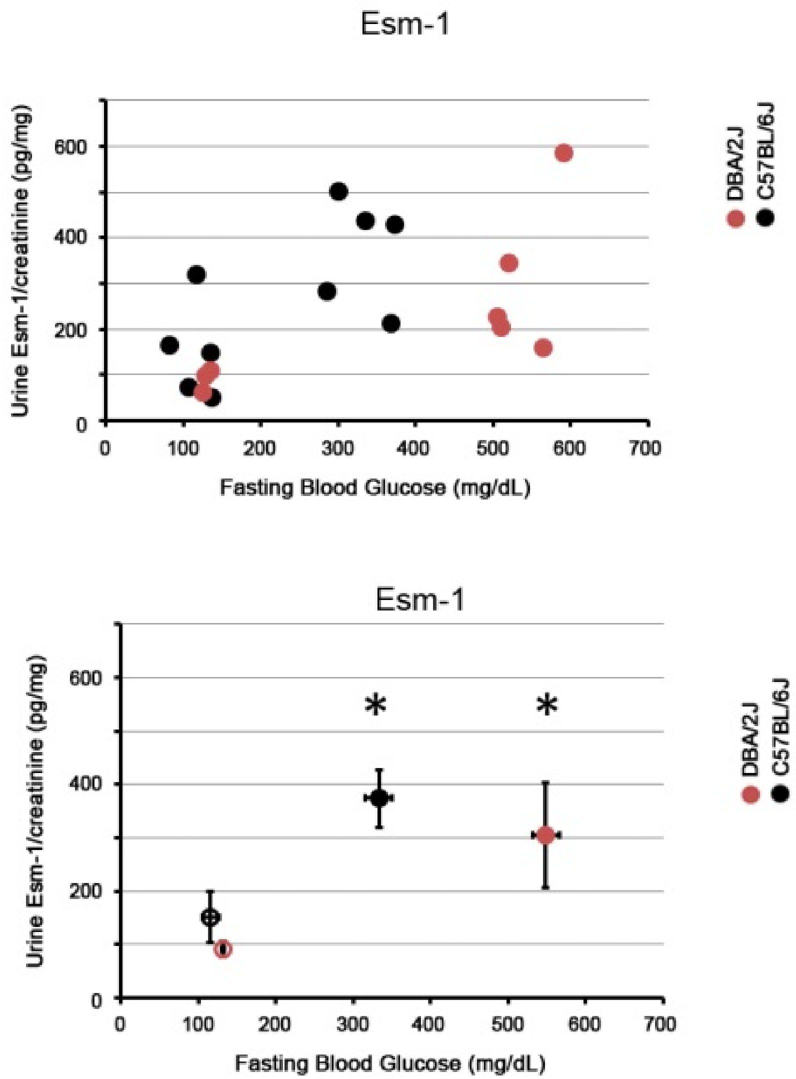

Figure 9: Esm-1 in urine with 4 weeks of diabetes. *, p-value < 0.05 vs. non-diabetic.

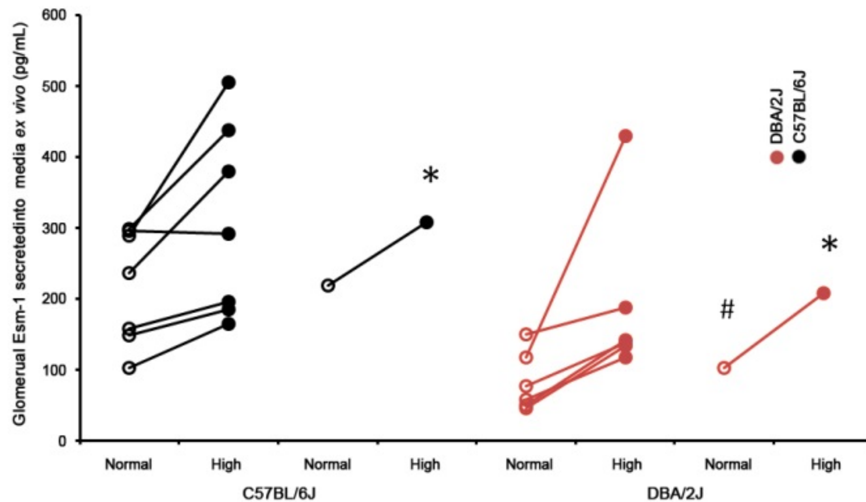

Figure 10: Esm-1 in glomeruli after 24 hours of normal or high glucose media. *, p-value < 0.05 vs. normal glucose. #, p-value < 0.05 vs. C57BL/6J mice.

Aim 3. Induce diabetes in DN-susceptible mice, over-express circulating Esm-1 and determine the contribution of Esm-1 to leukocyte infiltration, albuminuria, and the characteristic histologic changes of DN.

Results:

We have not successfully been able to over-express Esm-1 chronically despite the ability to increase expression of other secreted proteins (see **Figure 11**). Therefore, we are moving forward with an alternate experiment to knockout Esm-1 in endothelial cells and will perform similar experiments as proposed in the P&F application, but now comparing WT and KO mice rather than vehicle- or Esm-1 over-expressing mice.

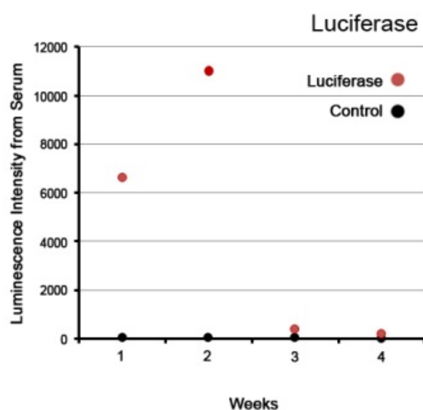

Figure 11: Validation of hydrodynamic tail vein injection for overexpression of secreted proteins in mice. Hydrodynamic injection of mice with luciferase and transposase expression plasmid. The serum luciferase activity was tested every week. As predicted, circulating luciferase persists at least 7 days, consistent with published literature[22]. HEK293T cell culture media with/without luciferase expression plasmid transfection was used as positive/negative controls, respectively (not shown).

3. Publications:

We have a manuscript in preparation from this work. Our plan is to submit to *Diabetes* within the next 1-2 months.

4. Project-Generated Resources

We have collected and stored tissue, serum, and urine samples in diabetic and non-diabetic DBA/2J and C57BL/6J mice. These samples will be available for future tests as needed or requested by colleagues upon acceptance of our manuscript on the role of Esm-1 in the diabetic kidney.

5. Collaborator/Consultant Update

The two consultants for the principal investigator (Drs. PJ Utz and Justin Annes) are still actively involved in the project. Additionally, we have added Dr. Mohammad Kiani (Temple University) to assist with in vitro functional assays. Dr. Ralf Adams (Max-Planck University) has also shipped Esm-1 floxed embryos for re-derivation of mice.

6. Vertebrate Animals

Our animal protocol has been active for the duration of the funding period, and there are no anticipated changes to the protocol.

7. Select Agent Research

Not applicable