DCC Mid-term Report (2013)

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Project title: Functional role of candidate genes emerging from GWAS in diabetic nephropathy

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Abstract: Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) worldwide. Although tight control of blood sugar can decrease the risk of diabetic kidney disease, many patients with adequate control develop DN, while others remain complication-free despite poor glycaemic control. Family studies point to a strong role for inherited factors in the development of DN, but the relevant genetic variants remain unknown, limiting our understanding of this devastating complication. Our long term goal is to discover the genetic factors that influence the risk of DN, with the hope that improved understanding of the disease will identify novel biological targets for therapeutic and preventive intervention. The Genetics of Nephropathy – an International Effort (GENIE) Consortium have recently completed the largest genome wide association study (GWAS) to date and meta-analysis of type 1 DN and ESRD. This Pilot application has two aims: Aim1 - To investigate the functional relevance of candidate genes from the GENIE GWAS: ERBB4 - encoding an epidermal growth factor receptor subfamily member; and AFF3 - an AFF transcription factor and type 1 diabetes candidate gene. Our preliminary data from renal cell models and expression analysis suggest a potentially important role for both of these genes in DN. We will investigate the putative role of these genes by either overexpressing or repressing levels in cell models that we have previously shown to mimic important aspects of the pathology of DN. These data will inform further investigations of differential gene expression and regulatory networks which may be awry in DN. Aim 2 – To investigate the FRMD3/rs1888747 variant previously reported to be associated with DN. Recent data from our collaborators suggest that FRMD3 gene expression is dysregulated in DN, potentially due to the rs1888747 promoter variant. We will investigate the impact of this variant on FRMD3 function in our established cellular models of DN. In summary, we aim to interrogate the emerging GWAS data, and investigate the underlying biological signature.

The specific aims of the current proposal were:

Specific Aim 1: To investigate the function of genes from GENIE GWAS in DN. We propose to test the roles of candidate genes arising from the GENIE GWAS, through interrogation of human samples, cell model systems, and coordination with existing renal expression datasets.

Specific Aim 2: To characterize previously associated DN gene - FRMD3. We propose to investigate the function of FRMD3 variant rs1888747 in the context of DN, and investigate the predicted relationship between FRMD3 and BMP pathway members in cell model systems.

Rationale and Relevance

Diabetic nephropathy (DN) is associated with significant morbidity and mortality in type 1 and type 2 diabetes mellitus. DN is a serious complication of diabetes and the leading cause of end-stage renal disease (ESRD) that requires dialysis treatment or kidney transplantation. Familial aggregation confirms the influence of inherited risk factors, but specific genes and variants are not yet known. Discovery of genetic variants responsible would identify pathways involved in the pathophysiology process, guiding development of new therapies and strategies

Specific Aim 1: To characterize associated genes from GENIE GWAS in DN

At present we have focused on Specific Aim 2 and have no significant updates to report for Specific Aim 1.

Specific Aim 2: To characterize associated DN gene - FRMD3

Background: FRMD3 (FERM domain-containing protein 3) encodes a member of the protein 4.1 superfamily, located on chromosome 9q and present in five splice variants. The FERM domain is a 30-kDa membrane-binding domain located at the N-terminus in the Protein 4.1 family homologues. FERM domains are found in a number of cytoskeletal-associated proteins that typically associate with various proteins at the interface between the plasma membrane and the cytoskeleton.

Recent work by our collaborator Matthias Kretzler at University of Michigan suggests that FRMD3 renal expression is down-regulated with severity of DN, and this is potentially due to the creation of a negative regulatory transcription factor binding site for homeodomain transcription factors (HOMFs) created by the SNP rs1888747 (C>G allele change) in the FRMD3 promoter, located approximately 2.4 Kbp upstream of the transcription start site. They identified a transcriptional regulatory module in the FRMD3 promoter consisting of four transcription factor binding sites (TFBSs) predicted to inhibit expression of FRMD3. These TFs include an inhibitory HOMF site created by the ‘G’ allele at the associated SNP site, and also

BRNF, BRN5 and GATA sites located nearby. Upon completion of this TFBS module by the 'G' allele, this module is predicted to inhibit FRMD3 promoter expression.

Interestingly, this promoter module was also identified in 22 bone morphogenetic protein (BMP) pathway family members whose expression is highly correlated with FRMD3 expression in renal biopsies of DN patients, suggesting co-regulation of BMPs with FRMD3. BMPs belong to the transforming growth factor-β (TGF-β) superfamily and exert reno-protective effects in adult kidneys.

Hypothesis 1*: Loss of renal FRMD3 expression has a negative effect on kidney function and drives DN progression*

Hypothesis 2*: The presence of the 'G' allele at the associated FRMD3 promoter SNP (rs1888747; C>G) creates a binding site for an unidentified member of the HOMF TF family, which acts to complete an inhibitory transcription factor module*

Hypothesis 3*: FRMD3 and BMP pathway genes are co-regulated. In the presence of the 'G' allele, FRMD3 expression is inhibited by HOMF TF binding leading to attenuation of BMP expression, therefore furthering DN progression.*

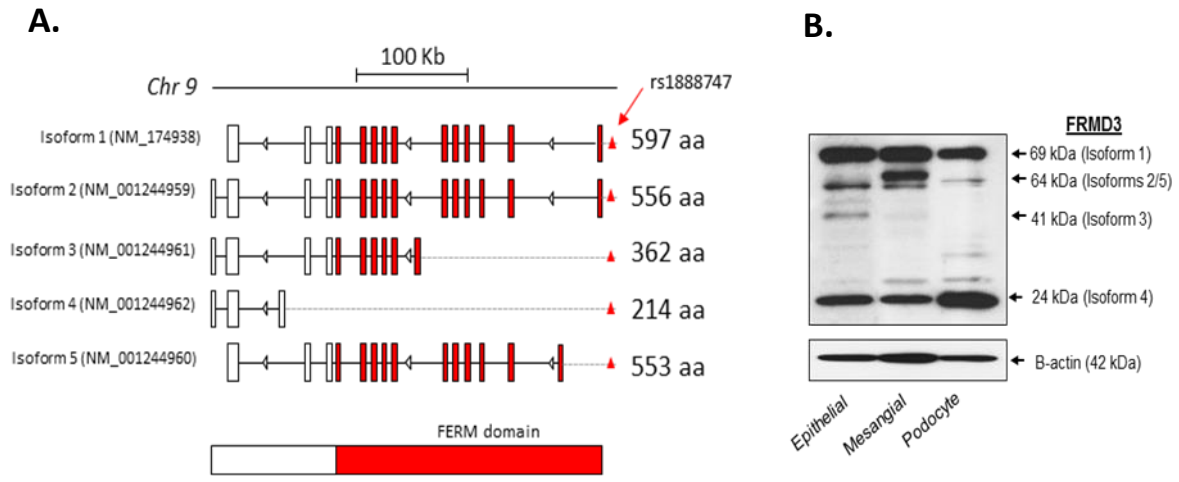
Progress towards stated aims

1. We will first investigate the functional role of *FRMD3* in cell model systems and the predicted relationship with BMP family members.

Results

FRMD3 renal expression – Aim: To characterise basal FRMD3 expression in human renal cells [renal epithelial (HK-2), primary human mesangial (hMC) and podocyte cells]. **Methods:** Western blot analysis was used to detect FRMD3 (protein 4.1O) expression in HK-2, primary hMC and podocyte cell lines. FRMD3 renal expression – **Results:** Schematic representation of five human FRMD3 transcript isoforms, and location of associated SNP rs1888747 (Fig 1A). FRMD3 protein products were detected in HK-2, primary hMC and podocyte lysates (Fig. 1B). FRMD3 antibody (Santa Cruz: sc-164431) detects protein isoforms at 69/64/41/24-kDa. **Conclusions:** FRMD3 is expressed in human renal epithelial, mesangial and podocyte cells. **Future directions:** We will use immunofluorescence microscopy to determine the subcellular localization of FRMD3 in these cells.

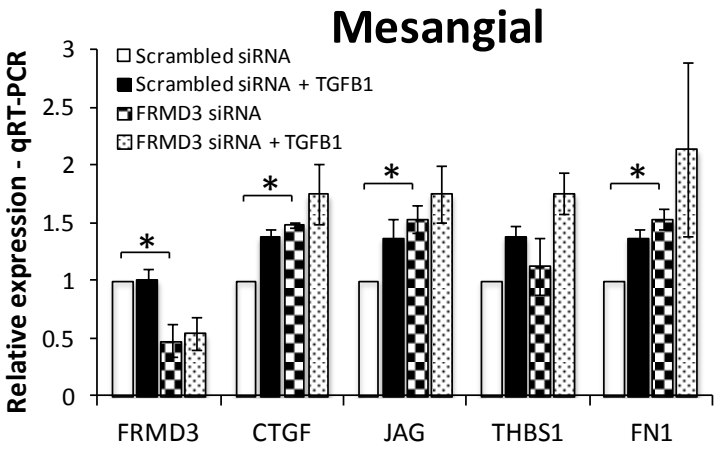
Fig 1.



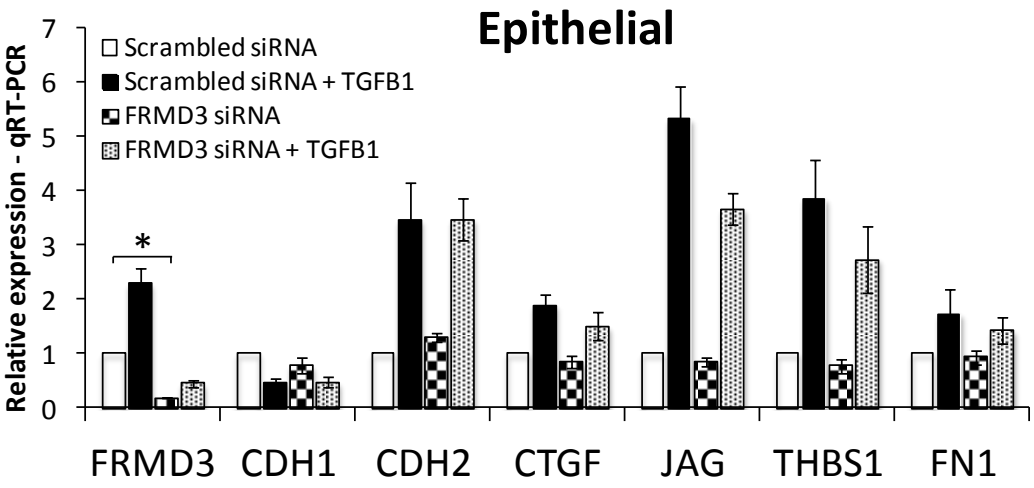
Impact of loss of FRMD3 – Background: We hypothesized that loss of FRMD3 expression may lead to DN by an increase in fibrosis and may enhance the fibrotic action of TGF-β1 in renal cell models. **Aim:** To measure markers of fibrosis in renal cell lines (hMC, HK-2, human podocyte) and slit diaphragm integrity markers in podocytes following transfection with FRMD3 siRNA (-/+TGF-β1). **Methods:** Markers of fibrosis were measured in cDNA extracted from cells previously starved for 24hr and transfected with FRMD3 siRNA (24hr), then stimulated (-/+ TGF-β1, 5ng/ml, 24hr). Slit diaphragm integrity of podocytes was measured by western blot analysis of podocin expression. **Results:** FRMD3 knockdown with siRNA was successful in all models and resulted in increased expression of fibrotic markers (CTGF, jagged-1, and fibronectin-1) in hMCs (Fig 2A). TGF-β1 stimulation significantly up-regulated FRMD3 expression in HK-2 cells only (Fig. 2B). FRMD3 protects against deleterious effects of TGF-β1 signalling on podocin expression in podocytes (Fig. 2C). Therefore FRMD3 may play an important role in maintenance of podocyte slit diaphragm integrity and podocyte effacement. **Conclusions:** Suppression of FRMD3 expression in mesangial cells leads to upregulation of markers of fibrosis and actin cytoskeleton rearrangements. Suppression of FRMD3 expression in podocyte cells leads to loss of podocin expression. **Future directions:** Confirm the mesangial cell real-time data at the protein level by western blot and continue investigations into effects of FRMD3 siRNA on mesangial cytoskeleton and podocyte podocin loss by immunofluorescence.

Fig 2.

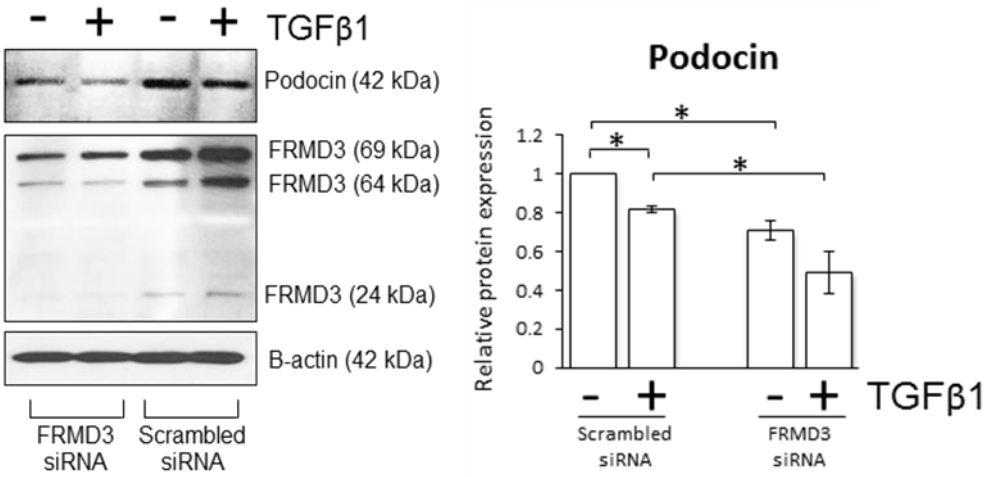
A.


Mesangial
Scrambled siRNA
Scrambled siRNA + TGFB1
FRMD3 siRNA
FRMD3 siRNA + TGFB1
Relative expression - qRT-PCR
0
0.5
1
1.5
2
2.5
3
*
FRMD3
CTGF
JAG
THBS1
FN1

B.

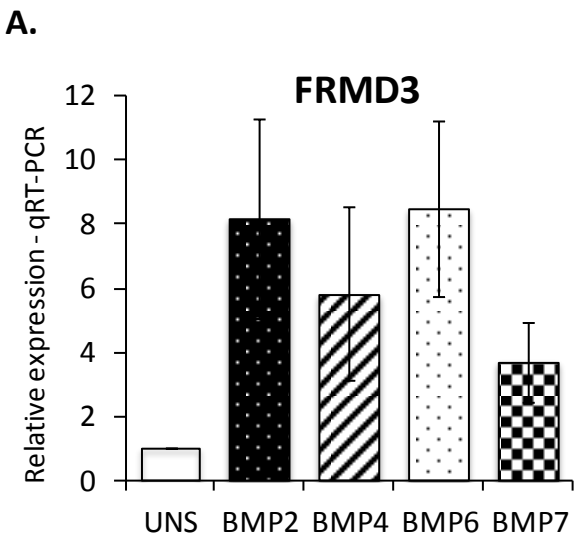

Epithelial
Scrambled siRNA
Scrambled siRNA + TGFB1
FRMD3 siRNA
FRMD3 siRNA + TGFB1
Relative expression - qRT-PCR
0
1
2
3
4
5
6
7
*
FRMD3
CDH1
CDH2
CTGF
JAG
THBS1
FN1

C.

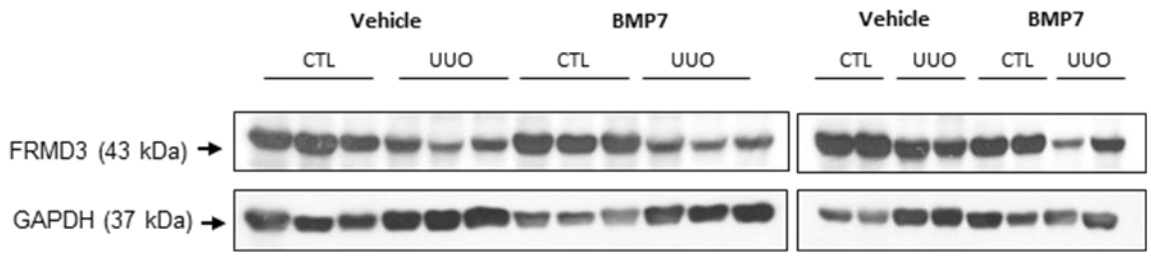

- + - + TGFβ1
Podocin (42 kDa)
FRMD3 (69 kDa)
FRMD3 (64 kDa)
FRMD3 (24 kDa)
B-actin (42 kDa)
FRMD3 siRNA
Scrambled siRNA
Podocin
Relative protein expression
0
0.2
0.4
0.6
0.8
1
1.2
*
*
*
- + - + TGFβ1
Scrambled siRNA
FRMD3 siRNA

Elucidation of the BMP-FRMD3 regulatory link - Background: *In silico* analysis of the FRMD3 promoter identified a TFBS module also present in BMP pathway family members indicating potential co-regulation between FRMD3 and the BMP pathway. **Aims:** To examine BMP effects on basal FRMD3 expression. **Methods:** Quantitative real time RT-PCR (Taqman) was carried out to detect FRMD3 expression levels in hMC cDNA post stimulation (24hr) with BMP-2, BMP-4, BMP-6 and BMP-7 (10ng/ml). **Results:** Stimulation of hMCs with BMP-2, BMP-4, BMP-6 and BMP-7 significantly increased FRMD3 expression (Fig. 3A.). Using material from an unpublished *in vivo* study from our group, we also measured renal FRMD3 expression in protein lysates derived from an *in vivo* model of chronic renal fibrosis (unilateral ureter obstruction), where control and UUO rats were administered either vehicle or BMP7. Here we observed significant loss of FRMD3 expression in UUO kidneys versus controls (Fig. 3B-C). We did not observe any FRMD3 responses to BMP7 in this model. We noted that in our human mesangial cell experiments, BMP7 was the least potent BMP to induce upregulation of FRMD3 (Fig. 3A). Therefore, it is possible that other BMPs (2, 4, 6) may induce upregulation of renal FRMD3 expression in the UUO model. Furthermore, while the UUO model primarily drives renal tubule fibrosis, an animal model where mesangial expansion is more predominant may be more beneficial for these studies. **Conclusions:** BMPs stimulate upregulation of FRMD3 expression in hMCs. FRMD3 expression is downregulated in UUO kidneys versus control kidneys. BMP7 does not restore the loss of FRMD3 expression in the UUO kidney. **Future directions:** Determine whether siRNA mediated FRMD3 silencing suppresses BMP signalling in hMCs and podocytes.

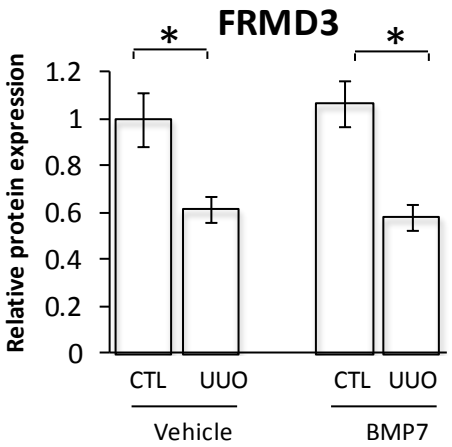
Fig 3.



B.



C.



Progress towards stated aims

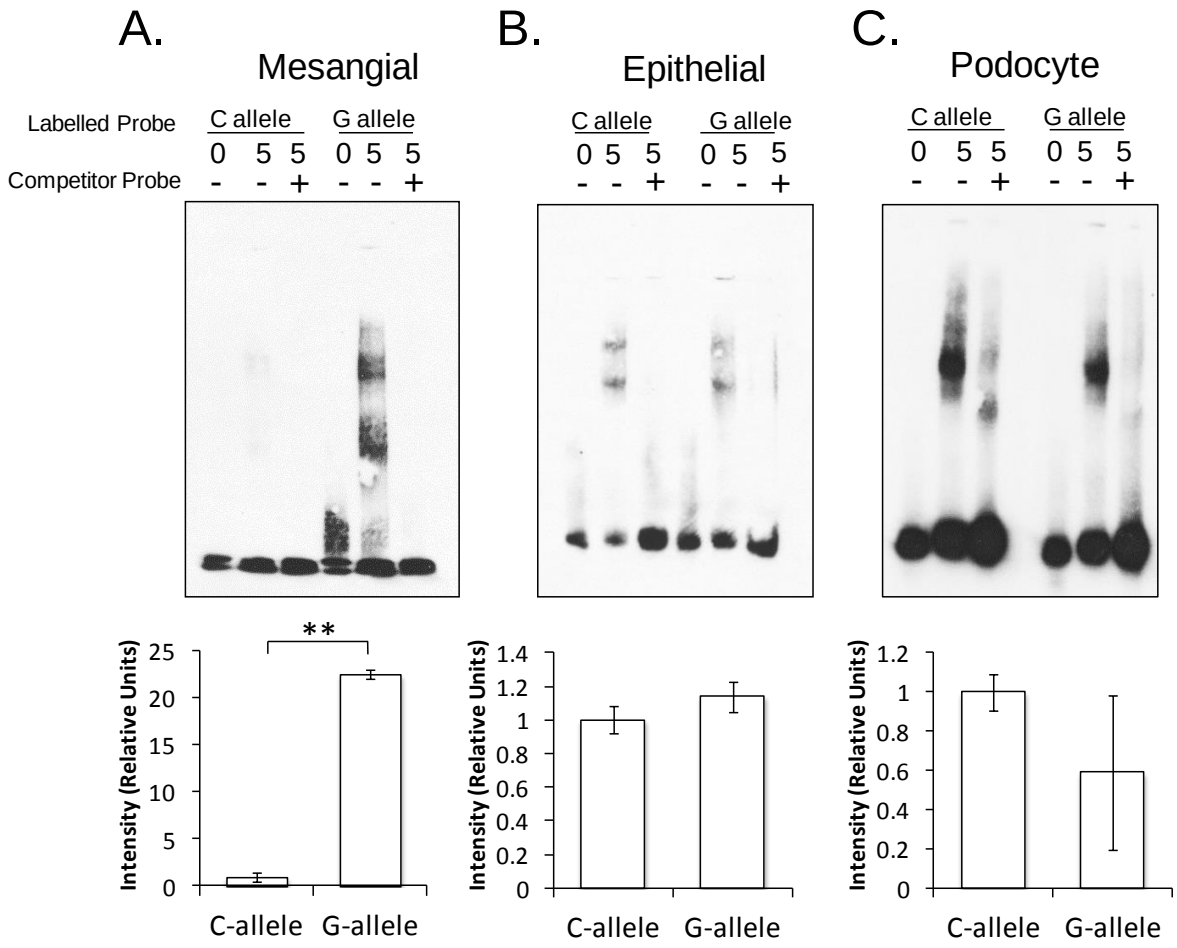
2. We will then determine the functional consequence of the wild type and mutated alleles of associated *FRMD3* SNP - rs1888747.

Results

Investigating the functional significance of SNP rs1888747 on TF binding - Background: Previously, our collaborator M. Kretzler utilised electrophoretic mobility shift assays (EMSAs) with C57BL/6J mice glomerular extracts and oligonucleotides corresponding to wild-type ('C' allele) and mutant ('G' allele) DNA sequences with complimentary 32-P-dCTP tagged strands. They reported that in the presence of the 'G' allele an increase in protein binding was observed. **Aim:** To replicate this experiment using biotin-labeled oligonucleotides for human sequences ('C' and 'G' allele) and nuclear extracts from human renal tubule epithelial (HK-2), primary hMC and podocyte cell lines (Fig. 3B-D.). **Methods:** Poly dI:dC was used to inhibit non-specific protein-DNA binding. Non-biotin labelled double stranded oligonucleotides corresponding to 'C' and 'G' allele sequences were used at 100-fold concentrations as competitors. **Results:** Primary hMC nuclear extract (5ug) binds preferentially to 'G' allele probes compared to 'C' allele probes (Fig. 4A). No significant difference in protein binding was observed with renal epithelial nuclear extracts (Fig. 4B-4C). While 'G' allele probes showed less

protein binding compared to 'C' allele probes in podocyte nuclear extract, these results were not statistically significant (Fig. 4C). Taken together, these data indicate that the SNP may potentially be functional in mesangial cells due to enhanced binding of an unknown transcription factor. **Conclusions:** EMSAs indicate that presence of the 'G' allele leads to preferential binding of transcription factors in human mesangial cells. **Future directions:** Determine what transcription factors are binding at the SNP site.

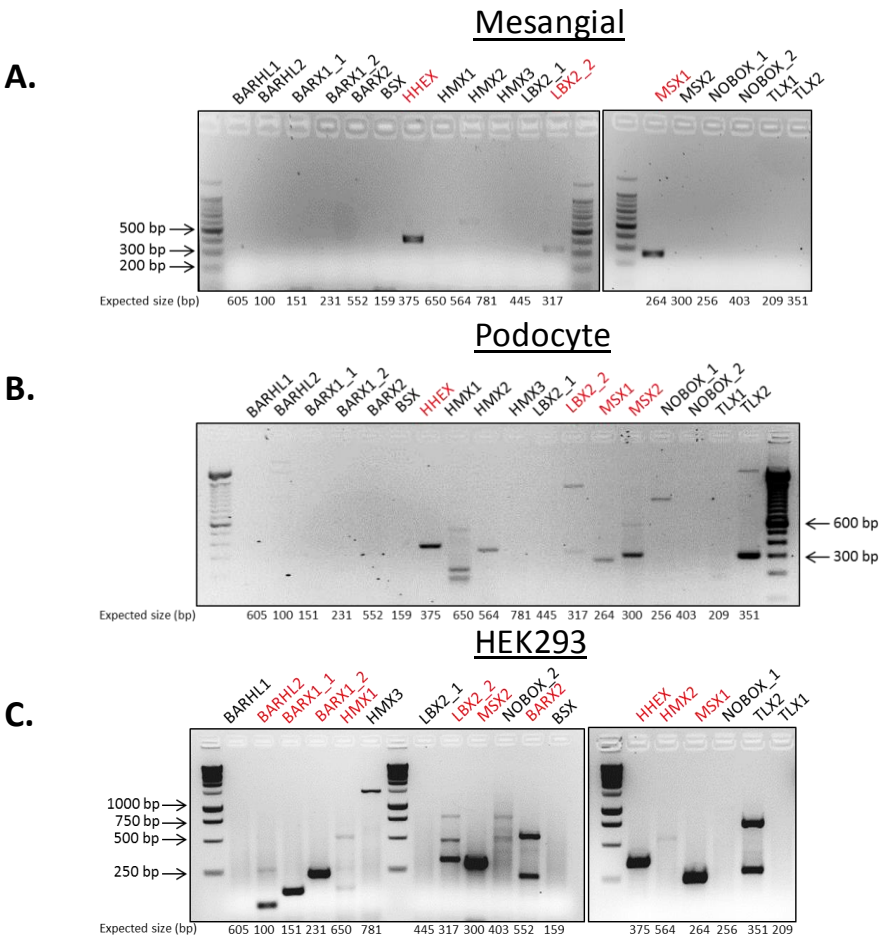
Fig 4.

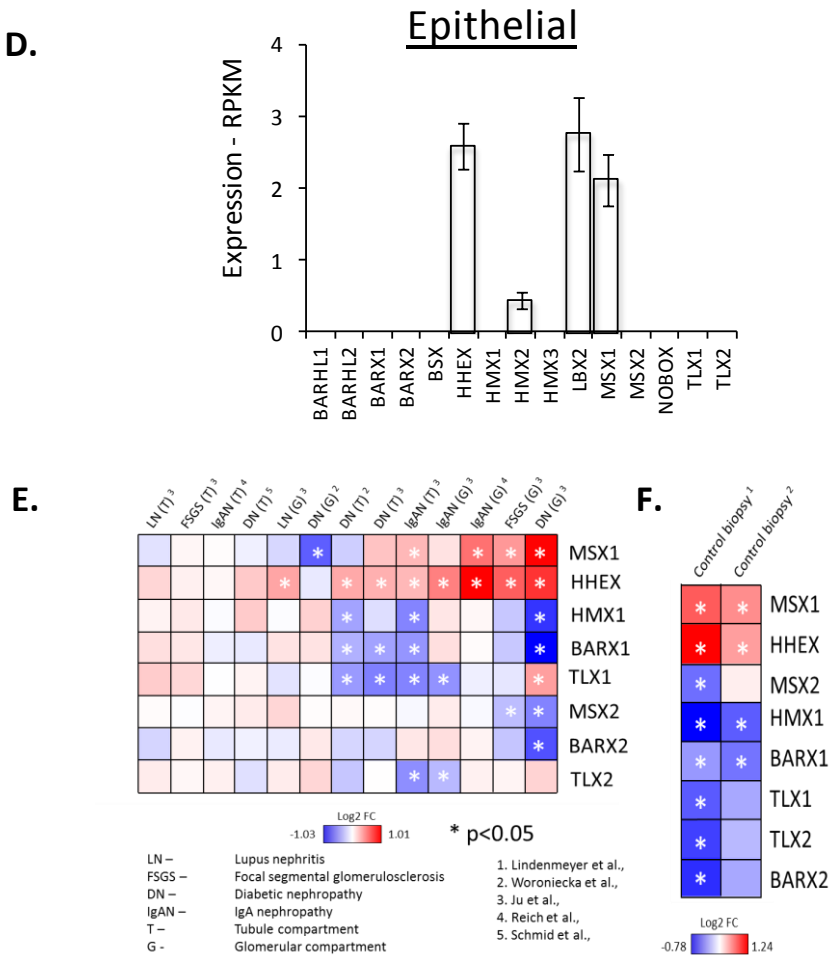


HOMF expression in renal cells - Background: Based on sequence analysis, homeodomain factors (HOMFs) are predicted to bind at the FRMD3 SNP site in the presence of the 'G' allele (rs1888747; C>G). **Aims:** To characterize HOMF expression in renal cells. **Methods:** Semi-quantitative PCR was carried out using hMC and podocyte cDNA to determine the expression of 18 predicted HOMF transcript isoforms. HEK293Ts were selected as a positive control cell line for HOMF expression based on a literature review. HOMF expression in epithelial cells (HK2) was determined using RNA-Seq data previously generated by our group. An online tissue expression microarray database (www.nephromine.org) was used to validate semi-quantitative PCR results by determining HOMF expression in microdissected renal biopsies from patients

with CKD versus controls. EMSA assays were used to determine specific HOMF interactions with 'C' and 'G' allele probes. **Results:** hMCs expressed HHEX, MSX1 and LBX2 isoform 2 (Fig. 5A). Podocytes expressed HHEX, MSX1, MSX2 and LBX2 isoform 2 (Fig. 5B). HK-2 cell RNA-Seq data indicated expression of HHEX, MSX1, LBX2 and HMX2 (Fig. 5D). Overall, HOMF transcript abundance in HK-2 cells was extremely low. Positive bands were observed for 10/18 HOMF transcript isoforms using HEK293T cDNA (Fig. 5C). Finally, analysis of HOMF transcript expression using the Nephromine resource identified HHEX and MSX1 as the predominant HOMFs upregulated in multiple CKD phenotypes including DN biopsies versus controls (Fig. 5E). In control biopsies HHEX and MSX1 expression was greater in the glomerular versus tubule compartments (Fig. 5F). **Conclusions:** HHEX and MSX1 are the prioritized HOMF candidates selected as potential binding partners at the SNP site. **Future directions:** We did not see any evidence of renal expression for several HOMFs. However, this may also be a PCR issue. Therefore, additional primer pairs will be designed to amplify these transcripts.

Fig 5.



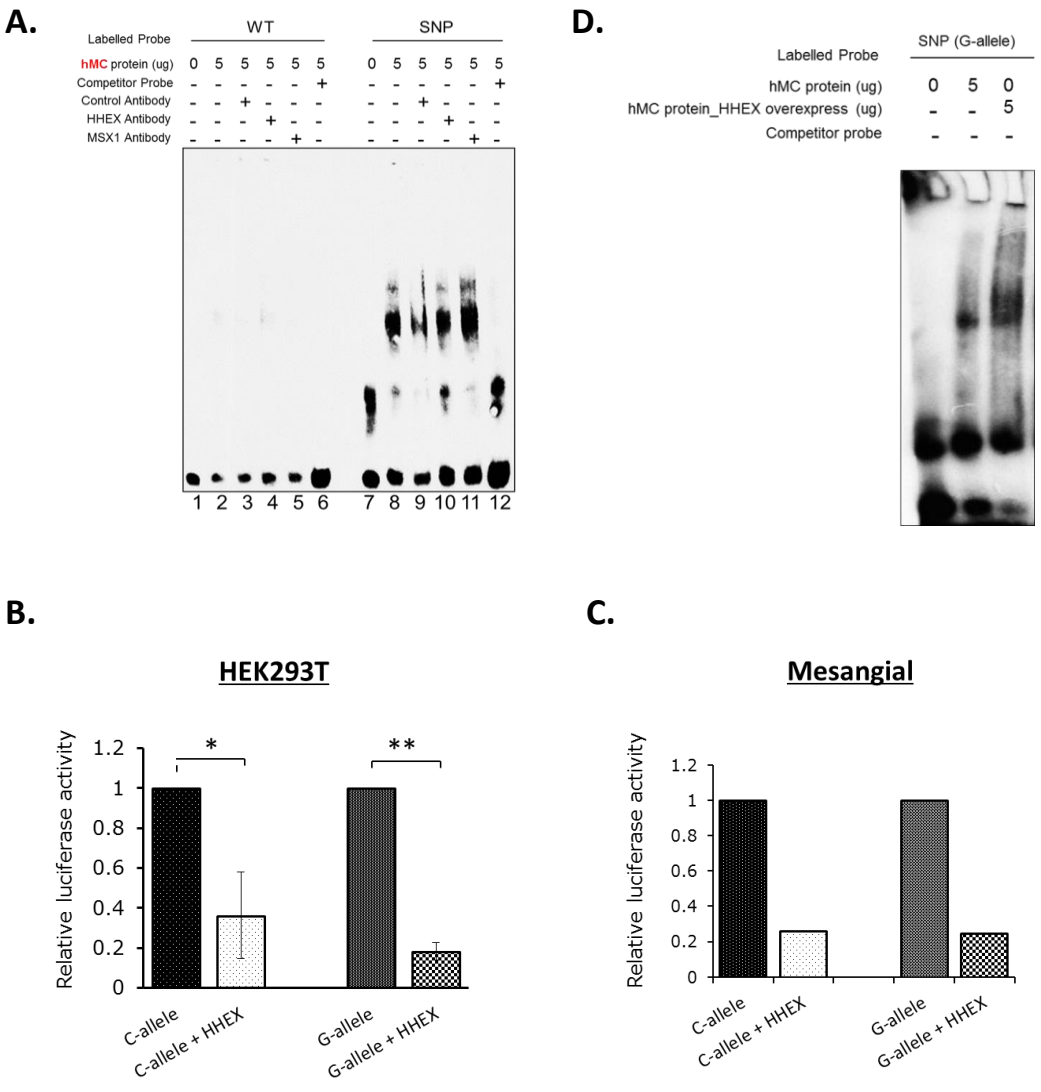


Legend: HOMF expression in published microarray datasets from the nephromine resource. **(E)** Red represents those HOMFs upregulated in CKD versus controls. **(F)** Red represents those HOMFs upregulated in glomerular versus tubule compartments of healthy donor biopsies.

HHEX binding at SNP site in FRMD3 promoter - Background: Our screening of HOMF expression in human renal cells (epithelial, mesangial and podocyte) indicate the primary candidates as binding partners at the SNP site are HHEX and MSX1. **Aims:** To determine which HOMFs bind at the SNP site. **Methods:** Three approaches were selected to determine whether HHEX or MSX1 are binding at the SNP site: (1) Antibody supershift assays using HHEX and MSX1 antibodies to investigate whether these antibodies selectively 'pull-up' the 'G' allele probe; (2) Generate full length FRMD3 promoters luciferase constructs corresponding to 'C' and 'G' allele, and determine whether HHEX or MSX1 overexpression preferentially suppresses the 'G' allele promoter; and (3) Perform an EMSA using mesangial cell nuclear extract overexpressing or silencing HHEX. **Results: (1)** EMSA super-shift assays with HHEX/MSX-1 antibodies showed no specific binding (Fig. 6A). *A limitation to consider here is that the lack of effect may be due to the specificity of the antibodies for supershift assays.* **(2)** In HEK293Ts (n=3) and hMCs (n=1) co-transfected with HHEX over-expressing vector and FRMD3 promoter luciferase constructs, a significant decrease in luciferase activity was observed upon HHEX overexpression, thus

confirming the inhibitory action of HHEX on FRMD3 expression (Fig. 6B-C). However, no significant differences in luciferase activity were observed between 'C' and 'G' promoter. *A limitation here is that there are multiple HOMF sites distributed along the FRMD3 promoter which may make it difficult to discern between these promoter constructs using a full promoter construct approach. In addition, overexpression of other TFs in the promoter module may be required to determine the SNP effect.* **(3)** Preliminary EMSA data using normal hMC nuclear extract versus hMC extract enriched for HHEX (using a HHEX overexpression plasmid) demonstrated increased binding to the 'G' allele probe (n=1; Fig. 6D). **Conclusions:** Preliminary EMSA data indicates that HHEX may be a potential binding partner at the SNP site. **Future directions:** Replication of EMSA data will be required to determine whether HHEX is indeed the binding partner at the SNP site. Unbiased ChiP screening may be required to determine the potential binding partners at this SNP site.

Fig 6.



Overall Project Conclusions

- FRMD3 expression is detectable in human kidney epithelial, mesangial and podocyte cells
- Suppression of FRMD3 expression upregulates markers of fibrosis in mesangial cells
- Suppression of FRMD3 expression leads to loss of podocin expression in podocytes
- BMPs 2,4,6, and 7 upregulate FRMD3 expression in hMCs
- FRMD3 expression is downregulated in UUO versus control rat kidneys
- EMSA assays indicate enhanced TF binding to the G allele in mesangial cells
- HOMF screening identifies HHEX and MSX1 as potential SNP site binding TFs
- HHEX suppresses FRMD3 promoter luciferase activity
- Preliminary EMSA data using HHEX suggests HHEX binds to the 'G' allele

Plans for the remainder of the year and completion of the project

- Characterization of FRMD3 renal subcellular localization by immunofluorescent microscopy
- Confirm mesangial cell real-time data at the protein level by western blot
- Examine mesangial and podocyte cell cytoskeletal arrangement and slit diaphragm integrity (-/+ FRMD3 siRNA)
- If above results are promising, perform RNA-Seq expression analysis of these cell models to determine global effects of loss of FRMD3 in the cells
- Examine the effects of FRMD3 silencing on BMP 2/4/6/7 expression in renal cells
- Design oligonucleotides for all remaining HOMFs to confirm presence/absence of these transcripts in renal cells
- Potential SNP-site binding partners may be determined by ChiP sequencing
- The importance of the 4 TFBS module incorporating the SNP may be examined in renal cells co-transfected with FRMD3 full promoter reporter luciferase vectors and HOMF over-expressing vectors in combination with GATA, BRNF or BRN5 over-expressing plasmids
- Project Aim 1 (investigate GENIE GWAS candidates AFF3 and ERBB4) will also be pursued using a similar approach to what has been employed for FRMD3. *Presently the AFF3 and ERBB4 candidate genes are being fine-mapped to determine where the precise genetic signal resides.*