Final Report

For the one year of funding provided by the AMDCC, we had two major aims. First, we wished to test the hypothesis that changes in microRNA (miR) expression could act as a marker for the presence and development of diabetic nephropathy. If miRs play a role in DN, then we would anticipate that alterations in miR expression would correlate temporally with disease occurrence. To test this hypothesis we analyzed miR expression by genome-wide miR expression profiling on RNA extracted from the kidneys of 10-week-old C57BLKS-db/db-eNOS-/- mice (db/db-eNOS-/- mice, (1)) and C57BLKS-db/db-eNOS+/+ (db/db mice) which are considered a valid model of diabetic nephropathy (2). eNOS-/- and C57BLKS mice were used as controls. db/db-eNOS-/- mice exhibit hyperglycemia by 6 to 8 weeks of age, functional nephropathic changes consistent with DN, such as proteinuria, by ten weeks of age and fulminant changes by 16 to 20 weeks. Indeed, microalbuminuria was observed in these 10 week old mice (not shown). db/db mice become diabetic in a similar time frame but exhibit milder kidney pathology when compared to age matched db/db-eNOS-/- mice (1), C57BLKS and eNOS-/- mice do not develop diabetes or DN. Neither strain exhibited microalbuminuria. When RNA samples from 10-week old db/db-eNOS-/-, db/db and C57BLKs mice (*n=3-5 per group*) were examine by genome–wide expression analysis using arrays to assess miR expression, it was immediately apparent that it is possible to distinguish between each group based on differences in miR expression (Fig. 1). We identified 125 miRs in analyzed based on sequences in miRbase 14 that are differentially expressed and can be divided into 5 groups (A-E) based on differences in expression observed between each strain analyzed (Fig. 1). miRs in group A are significantly down regulated in db/db-enos-/- mice which are diabetic and exhibit signs of DN, and exhibit a clear difference when compared to db/db mice that are diabetic but do not have signs of DN, as well as controls. Expression of miRs in group B was similar in db/db-enos-/- mice and db/db mice, but reduced when compared to C57BLKS mice. Expression of miRs in group C was similar in db/db- enos-/- mice and C57BLKS controls, but is reduced in db/db mice. miRs in groups D were up-regulated in db/db mice when compared to C57BLKS controls. However, db/db-eNOS-/- mice do not appear to be able to up-regulate these miRs to the same extent as db/db mice. miRs in group E were up-regulated in both db/db-eNOS-/- and db/db mice relative to C57BLKS mice, however these miRs are up-regulated to the greatest extent in db/db-eNOS-/-. When db/db-eNOS-/- mice were compared with control eNOS-/- mice (not shown), 98 miRs were differentially regulated. Similarly, when db/db mice were compared with control C57BLKS mice, 57 miRs were differentially regulated. This suggests that miR expression changes occur in response to hyperglycemia independently of changes due to the kidney injury inherent in DN, since at this time point db/db mice do not have DN.

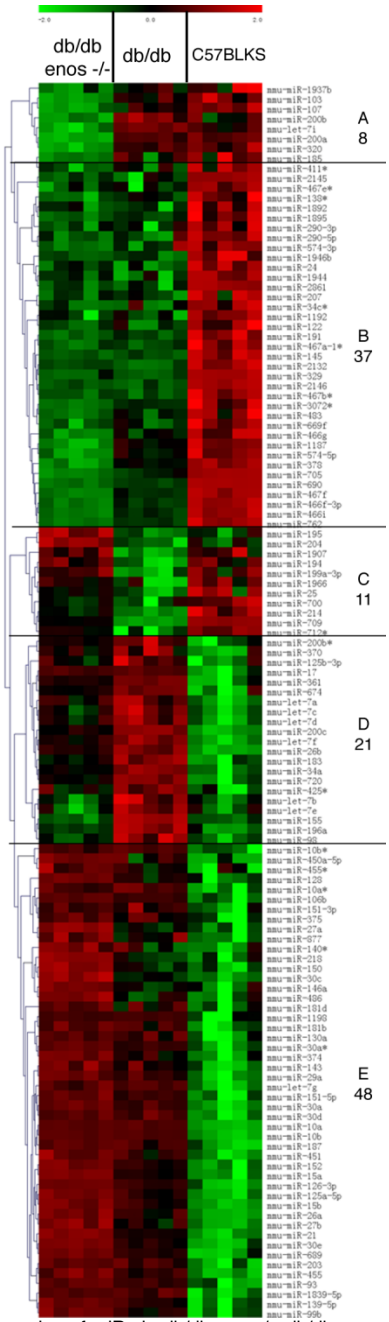

Fig. 1: Differential expression of miRs in db/db-enos-/-, db/db and C57BLKS mice at ten weeks of age. Groups (A-E) are indicated on the right along with the number of miRs in each group. Full size figure is available in the appendix.

Evidence that differential expression of miRs coincides with the onset of DN. We also performed expression profiling on 4 and 7-week old mice in addition to 10-week old mice (*n=3-5 per group)*. While at 10 weeks, 98 miRs were differentially expressed in db/db-eNOS-/- mice when compared to eNOS-/- controls, at 4 and 7-weeks only 35 and 33 miRs respectively were differentially regulated. This is similar to the differences in altered miR expression observed when samples from eNOS-/- mice were compared to C57BLKS mice where 36 and 39 miRs respectively were differentially expressed at 4 and 7 weeks. This suggests that differences in miR expression, perhaps as a result of hypertension in eNOS-/- mice exist prior to the development of hyperglycemia. While these differences in miR expression may predispose eNOS-/- mice to the development of DN, they clearly pre-exist DN. These data also indicate that the majority of changes in miR expression that occur as a result of disease are evident at 10 weeks, but are not yet extant at 7-weeks, as would be expected, since these mice do not develop protenuria until after 7-weeks. Indeed, when PCA analysis is performed on samples from 7 and 10-week old mice it can be seen that there is less variation between the 7-week samples when compared with the 10-week samples (Fig. 2). Moreover, changes in miR expression at 10 weeks are specifically associated with diabetic strains that eventually develop DN, db/db and db/db-eNOS-/-, but not in controls strains such as eNOS-/- (Fig. 2).

In addition, we have collected samples from db/db-eNOS-/-, db/db eNOS-/- and C57BLKS at 14, 21 and 28 weeks of age, and are in the process of examining miR expression in kidneys from these animals in oder to extend our time course.

Analysis of miR expression data. To understand our miR expression data further, we conducted an intensive literature review and bioinformatic analysis of miR that were differentially expressed in 10-week old db/db-eNONS-/- mice and age matched controls. We focused on miRs that exhibited at least a 2-fold change in expression relative to C57BLKS controls. Recent publications summarized in (3) have indicated a potential role for 8 miRs in DN (miR-21, miR-93, miR-377, miR-192, miR-216a, miR-141, miR-200a and miR-29 family members. However, we observed that only miR-21 (6 and 3-fold increased), miR-29a (3-fold increased), miR-29c (9 and 2-fold increased) and miR-200a (3-fold decreased) were differentially expressed in 10-week old db/db-eNOS-/- mice compared to age matched db/db, or C57BLKS controls respectively. These observations were confirmed by PCR. In contrast to published reports we did not observe any significant difference in expression of miR-93, miR-377 or miR-192, miR-216a, or miR-141 in our model using 10-week old db/db-eNOS-/- mice when compared with age matched controls. This may be related to the time points analyzed, differences between our in vivo studies and in vitro assays or the difference between hyperglycemia and DN.

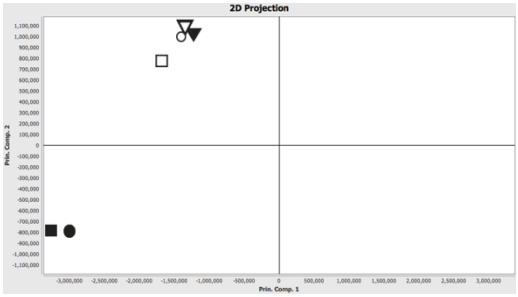

Fig. 2. PCA analysis. Kidneys from db/db-eNOS-/- mice (circles), db/db mice (squares), and eNOS-/- control mice (inverted triangles) were analyzed by array at 7 (open symbols) and 10 weeks (closed symbols) of age. Changes in miR expression over time were examined by PCA as described. PCA 1 (x-axis) and 2 (y-axis) account for 98% of variation. eNOS-/- and C57BLKS mice were similar (not shown).

To confirm that the miR expression changes that we observed were relevant to human DN, we initiated a collaboration with Dr. Marcus Bitzer who has examined DN in PIMA Indians with early DN. This population exhibits a high rate of type 2 diabetes and DN (NIDDK data repository, http://diabetes.niddk.nih.gov/dm/pubs/pima/index.html) (4). Dr. Bitzer has been able to demonstrate that miR-21 expression is abundant in glomeruli and associated with ACR in human diabetic nephropathy. Indeed, miR-21 was noted to be differentially expressed in renal biopsies from DN patients by others {Krupa, 2010 #11773}. More recently, similar results were obtained for miR-200a (Dr. Bitzer, personnel communication). Thus, the alterations in miR expression observed in mice recapitulate alterations observed in humans with DN, for both miRs examined. Thus we are able to generate candidate biomarkers in a m ouse model of DN, and these miRexpression changes appear to be conserved in humans with DN.

Our second major aim was to begin to identify pathways and targets of miRs that are differentially expressed in DN in order to dissect pathology and potentially identify novel therapeutic targets. Bioinformatics suggest that several of the differentially expressed miRs identified in mice with DN play roles in processes related to DN. For example, miR-21 levels are increased in cardiac fibroblasts after heart damage leading to an increase in ERK-MAP kinase activity through inhibition of Spry1 thereby regulating fibroblast survival, and growth factor secretion which in turn affects the extent of interstitial fibrosis (5). TGF-b and BMP signaling appears to promote expression of mature miR-21 by promoting the processing of primary transcripts of miR-21 (pri-miR-21) into precursor miR-21 (pre-miR-21) by Drosha (6). TGF-b levels are increased in renal cells such as mesangial cells under high glucose conditions, and TGF-b can regulate production of extracellular matrix proteins leading to fibrosis (7-9). Indeed, major characteristics of DN include glomerular basement-membrane thickening, mesangial expansion and hypertrophy, and an accumulation of extracellular matrix proteins resulting in fibrosis (9). We suggest therefore that up-regulation of miR-21, observed in 10 week old mice (Fig. 1), may play a central role in controlling glomerular fibrosis. miR-200a and mir-200c are also involved in TGF-b mediated signaling, and their down-regulation by TGF-b may contribute to epithelia-mesencymal transition (EMT) (10-12). miR-192 has been reported to be a critical downstream mediator of TGF-beta/Smad3 signaling in the development of renal fibrosis (13) and is overexpressed in kidney from db/db-eNOS-/- mice when compared with db/db mice. Similarly, miR-215 mediates TGF-b dependent changes in adhesion molecule expression in the kidney, and is expressed at higher levels in db/db-eNOS-/- mice (Fig. 1). miR-27b negatively regulates PPARg in adipocytes (14), a molecule that is well-known to regulate expression of genes that affect the development of diabetic complications. Indeed, PPAR-g is known to antagonize the effects of TGF-b (15-18). Up-regulation of miR-27b, (Fig. 1), may therefore contribute to DN by down-regulating PPARg. Thus the data we have generated shows an enrichment for miRs that are involved in TGF-b signaling, a known pathway of DN. These data therefore support the idea that miR expression profiling leads to identification of pathways involved in DN and may therefore lead to the discovery of new pathways of regulated gene expression that could be exploited for therapeutic intervention in DN.

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